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Investigations into mating disruption, delayed mating, and multiple mating in oriental beetle, Anomala orientalis (Waterhouse) (Coleoptera: Scarabaeidae).

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INVESTIGATIONS INTO MATING DISRUPTION, DELAYED MATING, AND MULTIPLE MATING IN ORIENTAL BEETLE, *ANOMALA ORIENTALIS* (WATERHOUSE) (COLEOPTERA: SCARABAEIDAE)

A Dissertation Presented

by

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ABSTRACT

INVESTIGATIONS INTO MATING DISRUPTION, DELAYED MATING, AND MULTIPLE MATING IN ORIENTAL BEETLE, *ANOMALA ORIENTALIS* (WATERHOUSE) (COLEOPTERA: SCARABAEIDAE)

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Oriental beetle, *Anomala orientalis* (Coleoptera: Scarabaeidae), is a pest of turf, ornamentals, and several crops, including cranberry; damage is caused by larval feeding on plant roots. Imidacloprid—the only new pesticide registered against scarabs in cranberry since the ban on organochlorines in the 1970s—is expensive and relatively ineffective against later instar larvae. I evaluated the potential management of oriental beetle in cranberry by mating disruption using female sex pheromone deployed from wax disks. I found strong trap shutdown in mating disruption treatments. Because attempts to disrupt mating may be compromised if males eventually find females, I studied the effects of delayed mating on female reproductive output in the lab. Females were relatively resilient to mating delay, exhibiting only a gradual decline in fecundity with increased age at mating. Thus, in order for mating disruption to be a successful management tool, mating must be prevented rather than delayed. I deployed tethered virgin females deployed in the field and found that mating is indeed largely prevented in

pheromone-treated bogs. These results demonstrate the strong potential of mating disruption for management of oriental beetle in cranberry.

I also studied relative fertilization success between males in successive matings of the same female. Characters of the first male to mate—including male body size and genitalia morphology—had primacy in influencing relative paternity, an uncommon pattern in sperm precedence studies. Surprisingly, relative paternity of the first male was inversely correlated with his size. Because this result is at odds with the general positive relationship between male size and reproductive success in insects, I hypothesized that larger males experience greater lifetime reproductive success when mating multiply. I then examined how male size and mating history impacted reproduction. Only for smaller males was reproductive output reduced in successive matings. These results suggest that after their first mating, smaller males must either compete using a lower quality ejaculate or submit to a longer refractory period to replenish ejaculate reserves. Thus, the sexually selected advantage of smaller males in their first mating is apparently balanced by lower lifetime reproductive potential relative to larger males.

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CHAPTER 1

INTRODUCTION

Classification and origin of oriental beetle

Controversy and confusion have surrounded the generic placement of oriental beetle, owing to differing generic concepts among taxonomists as well as a poor taxonomic foundation of the Anomalini (Potts 1974; Jameson et al. 2003). Oriental beetle was first described in the genus *Phyllopertha* Stephens by Waterhouse (1875) and has since been transferred among the genera or subgenera *Blitopertha* Reitter, *Anomala* Samouelle, and *Exomala* Reitter. Oriental beetle was consistently referred to as *Anomala orientalis* (especially in the American literature; e.g., Dunbar and Beard 1975; Baker 1986; Staines 1986) and occasionally *Blitopertha orientalis* in literature from Japan (e.g., Leal 1993) until Baraud (1991) revised the two genera and elevated *Exomala* from a subgenus of *Blitopertha* to generic rank. Following Baraud's revision (which was based primarily on morphology of the male genitalia), oriental beetle has been variously referred to as either *Anomala orientalis* (e.g., Cowles and Villani 1996; Grewal et al. 2002) or, more often, *Exomala orientalis* (e.g., Leal et al. 1994; Alm et al. 1999; Facundo et al. 1999a,b; Choo et al. 2002a,b; Polavarapu et al. 2002; Cowles 2003; Sciarappa et al. 2005). At least one recent publication (Yokoyama et al. 2003) retained the binomial *Blitopertha orientalis*.

Anomala comprises about 180 New World and 800 Old World species, several of which are economically important, which has contributed to interest in the group. However, the genus has yet to receive comprehensive taxonomic treatment (Jameson et

al. 2003). Piatella and Sabatinelli (1994) argued that the Anomalini lacks a modern systematic foundation and *Exomala* is probably polyphyletic. Moreover, Jameson et al. (2003) suggested that although *Anomala* is probably paraphyletic and that many species would be more appropriately placed in other genera, *Exomala* is poorly characterized and may be within the scope of the genus *Anomala*. Therefore, I follow Piatella and Sabatinelli (1994) and Jameson et al. (2003) and retain the more conservative combination *Anomala orientalis*—at least until a more comprehensive taxonomic treatment of *Anomala* is complete.

The region of origin of oriental beetle has been the subject of some confusion, as well. For example, Wolters (1932) stated that the species is native to Japan and Korea, and Tashiro (1987) said that it is probably native to the Philippines; Vittum, Villani, and Tashiro (1999) later pointed to Japan as the likely native region of oriental beetle. Indeed, the species was first described based on individuals collected in Japan (Waterhouse 1875), and oriental beetle's introduction to the mainland United States was likely via infested nursery stock from Japan (Friend 1929). However, I am aware of no direct evidence that links the evolutionary origin of oriental beetle to Japan or any other particular region. It is notable that of several biological control agents imported to Hawaii from throughout Asia, only those taken from the Philippines have been effective (Wolters 1932; see below). It seems that the region of origin of oriental beetle will remain a mystery perhaps until a DNA fingerprinting study is performed to work out the relationships among beetles collected from throughout Asia.

Pest status of oriental beetle in the United States

Oriental beetle was first recorded on the United States mainland in a New Haven, Connecticut nursery sometime before 1920 (Britton 1925) and was introduced to the Hawaiian island of Oahu sometime before 1908 (Wolters 1932). Oriental beetle is now known to occur in New Hampshire, Massachusetts, Rhode Island, Connecticut, New York, New Jersey, Pennsylvania, Delaware, Maryland, Virginia, North Carolina, Ohio, and Hawaii; individuals have also been found in Tennessee, West Virginia, Maine, and South Carolina, but it is not clear whether established populations exist in these states (Alm et al. 1999). Much of the population throughout the Northeast is probably not continuous; infested nursery stock is suspected to be the major route of dispersal (Alm et al. 1999).

Oriental beetle has been a problematic pest of turfgrass and ornamentals in the Northeast U.S. since its introduction, and has more recently been reported in crops such as blueberries (Polavarapu 1996; Polavarapu et al. 2002), cranberries (Dunn and Averill 1996; Averill and Sylvia 1998), strawberries, raspberries, peaches, and sweet potatoes (Polavarapu et al. 2002). In Hawaii, grubs have been pests of sugarcane (Wolters 1932; Bianchi 1935). Adult females may occasionally feed on flowers of various ornamental plants (including roses, hollyhocks, phlox, dahlias, and Japanese irises), but they are not considered pests in this life stage (Friend 1929; Hallock 1933); however, feeding by the larvae can cause considerable damage to the roots of many plant species. In the Northeast, most individuals in turf overwinter as third instar larvae, pupate the following spring, and reproduce by late July or early August (Vittum et al. 1999); however, as many as 15% of individuals undergo a two year life cycle in which larvae

overwinter as second instars, then overwinter the following year as third instars. A two-year life cycle apparently occurs for a small portion of the population in cranberry as well (personal observation).

Oriental beetle has been erroneously considered a minor pest in many systems, probably because adults are often relatively inconspicuous, and the larvae are indistinguishable from Japanese beetle (*Popilla japonica* Newman) larvae without magnification (Vittum et al. 1999). In mixed populations of the two species, damage from oriental beetle grubs is still often attributed solely to Japanese beetle (Vittum et al. 1999), despite earlier efforts to educate the public (Adams 1949). The second billing pest status of oriental beetle relative to Japanese beetle is also likely perpetuated by the fact that the distribution of Japanese beetle in the U.S. is more widespread. First recorded in the U.S. (near Riverton, New Jersey) only four years earlier than oriental beetle was found in Connecticut (Potter and Held 2002), Japanese beetle is now established over a considerably larger area than oriental beetle (Vittum et al. 1999). The dramatic difference in range is probably at least partly a function of the limited dispersal tendencies of oriental beetle. Facundo et al. (1999a) observed females to generally call, mate, and deposit eggs within a very small radius from their point of emergence from the soil. Female dispersal might only be associated with the seeking of floral feeding sites following the first bout of egg laying, in which most of the eggs are laid (Facundo 1997).

Potential management options for oriental beetle, with particular reference to cranberry

Residues from the organochlorine insecticides dieldrin and aldrin provided effective control of all scarab pests of cranberry for decades after the pesticides were banned in the 1970s (Averill and Sylvia 1998). No new pesticides had replaced the organochlorines for use against soil insects until imidacloprid was approved for use in cranberry in 2004. Although imidacloprid is effective against early instars, it is of little use against later (second and especially third instar) grub stages (Potter 1998; Koppenhöfer et al. 2002) and, therefore, must be applied prophylactically, before widespread damage to the crop is evident. Further, imidacloprid is expensive and inhibits host location for parasitoids of white grubs (Rogers and Potter 2003). The efficacy of imidacloprid against scarab pests in cranberry has yet to be determined.

Averill and Sylvia (1998) suggested that a summer flood would likely be effective in suppressing oriental beetle, as has been demonstrated for two other scarab pests: cranberry root grub (*Lichnanthe vulpina* Hentz) and cranberry white grub (*Phyllophaga anxia* LeConte). Although flooding bogs from mid-May through mid-July usually results in ca. 90% control of cranberry root grub (a wetland-adapted cranberry specialist), summer flooding also destroys the crop for that year. Yield in the year following a summer flood treatment may not rebound completely, and retreatment may be necessary within 5-10 years. Complete or partial bog renovation is another potential, albeit costly, option for management of scarab grubs in cranberry.

Several introduced parasitoid wasps might have some value in management of oriental beetle. On the Hawaiian island of Oahu, oriental beetle was a serious pest of sugarcane until a parasitoid wasp (*Scolia manilae* Ashmead) was introduced as a

biological control agent. *S. manilae* was so effective that oriental beetle is no longer a problem in Hawaiian sugarcane (Bianchi 1935; Vittum et al.1999). However, attempts to control Japanese beetle near Philadelphia with introduced *S. manilae* failed because the wasp did not survive the winters (Britton 1925). Two other hymenopteran parasitoids of scarabs (*Tiphia vernalis* and *T. popilliavora*) were introduced in the eastern U.S. to control Japanese beetle (King 1931). These wasps initially showed high levels of parasitism and good establishment, but distribution at least of *T. vernalis* ultimately declined (Ladd and McCabe 1966). *T. vernalis* apparently is still established across much of the range of Japanese beetle in the U.S. (Rogers and Potter 2003) and has been found parasitizing oriental beetle grubs in the field (Reding and Klein 2001), but its current or potential role in management of oriental beetle has yet to be evaluated. It would seem that successful management with these parasitoids in traditional agricultural systems would only be achieved by augmentation biological control, which would be costly, not to mention incompatible with most traditional insecticide use.

The use of entomopathogenic nematodes may be a more economical biocontrol option, since nematodes can be easily applied in cranberry through the chemigation system and then watered in to maintain a soil moisture level that favors survival and infection. While the use of nematodes for management of oriental beetle in cranberry remains unexplored, trials with heterorhabditid and steinernematid nematodes in turf have shown varying success (Alm et al. 1992; Yeh and Alm 1995; Choo et al. 2002a; Grewal et al. 2002; Lee et al. 2002); however, nematode treatments have generally not been as effective as traditional pesticides. A recently discovered, highly pathogenic nematode (*Steinernema scarabaei*) can exhibit nearly 100% mortality against third

instar oriental beetle grubs in the lab and field (Koppenhöfer and Fuzy 2003). Despite its great promise, however, difficulties with rearing this nematode currently limit its use.

A few other pathogens that infect oriental beetle are worth mentioning. Choo et al. (2002a) demonstrated moderate reduction of grub populations in turf using *Beauveria brongniartii*. Unfortunately, native isolates of this pathogen are not available in the U.S.; moreover, application of the fungi in larval habitats would be difficult, although autodissemination via modified pheromone traps might be possible (see Klein and Lacey 1999). Oriental beetle grubs are susceptible to milky disease, but it is unclear whether the causal organism(s) differs from the *Bacillus* bacteria that cause milky disease in Japanese beetle (but see Yokoyama et al. 2003). In any event, effective use of the bacteria as a biocontrol agent in the field has yet to be demonstrated (Vittum et al. 1999). The facultative pathogen *Bacillus thuringiensis* serovar *japonensis* (Btj) strain Buibui is specific to scarabs and capable of severely reducing grub populations in turf (Alm et al. 1997), but this strain is no longer commercially available (Koppenhöfer et al. 2002).

A laboratory study (Cowles and Villani 1996) and a field study in turf (Cowles et al. 1999) have demonstrated the effectiveness of an insect growth regulator (halofenozide) against oriental beetle, but it may be less effective than imidacloprid—at least against early instars. Broadway and Villani (1995) assessed the impact of ingestion of serine proteinase inhibitors in several species, but observed no mortality effects for generalist root-feeding scarabs, including oriental beetle.

Finally, determination of the major components of the female sex pheromone of oriental beetle (Leal 1993; Leal et al. 1994; Zhang et al. 1994) has opened the possibility of management of oriental beetle using semiochemicals. Recent progress has been made in the development of mating disruption of oriental beetle in blueberry and ornamentals (Polavarapu et al. 2002; Sciarappa et al. 2005), in turf (A. Koppenhöfer et al. 2005), and now cranberry (see Chapter 2).

My research on mating disruption and delayed mating in oriental beetle

Several aspects of the biology and ecology of oriental beetle make this species conducive to management by mating disruption, which I evaluate in Chapter 2. For example, males are responsive to pheromone 24 hours per day (Facundo et al. 1999b) and posses limited potential for remating (Chapter 5). Further, both males and females have limited dispersal tendencies (Facundo et al. 1999a). My results from a two-year study demonstrate the strong potential for mating disruption of oriental beetle in cranberry. Moreover, I show that my mating disruption treatments largely prevent and do not simply delay mating in oriental beetle.

I study the potential importance of the effects of delayed mating on reproductive output in oriental beetle in Chapter 3. The goal of mating disruption is to prevent mating by interference with pheromonal communication between the sexes. However, inhibiting the orientation ability of males may only result in a delay of mating for some females, in part because some males may find females by chance alone, if given enough time. Nevertheless, even if a portion of the females within a population mate eventually, management by mating disruption may still be effective if delayed mating

results in an appreciable reduction in the number of viable offspring females produce. In oriental beetle, I found that females are relatively resilient to mating delay. I discuss other behavioral and ecological variables that may contribute to the promise of mating disruption despite the resilience in this species to the potential negative impacts of increased age at mating (see Chapter 3).

Reproductive behavior and ecology of oriental beetle and my research on mating behavior and sexual selection

Facundo and colleagues (Facundo 1997; Facundo et al. 1999a) characterized the reproductive behavior and ecology of oriental beetle, which I describe here along with my own observations. Virgin females begin calling (i.e. raising the abdomen and releasing sex pheromone) immediately upon emerging from the soil but are never observed to call after mating. The first male to reach a female mates with her, with no overt pre-copulatory courtship behavior. In the lab, mated females burrow into soil to lay eggs and do not surface until most if not all of their eggs have been laid (Bianchi 1935; E.J.W., unpublished data). If a second male attempts to mate with the female before she re-enters the soil, she vigorously waives her hind legs, which functions to inhibit the male's ability to mount her; she also apparently prevents the male from achieving intromission[1] (personal observation), at least initially. Most rejection attempts are unsuccessful and the female eventually acquiesces. Twice-mated females produce no more eggs than once-mated or unmated females, and mating twice does not increase fecundity, which suggests that females derive no benefit from subsequent

[1] (Eberhard 2002) stated that in all of the beetle species whose mating he has observed (including 16 families), the male is clearly unable to intromit until the female opens the tip of her abdomen.

mating after successful insemination—other than perhaps increased genetic diversity among progeny (see Jennions and Petrie 2000) or insurance against an infertile mate.

The mating behavior and ecology of oriental beetle offer the opportunity to address some interesting evolutionary questions regarding sperm precedence (i.e. the relative fertilization success among males in successive matings of the same female) and multiple mating in both males and females. I explore some of these questions in the last two chapters of my dissertation.

Because females lack direct pre-copulatory mate choice and mating pairs exhibit no pre-copulatory courtship behavior, post-copulatory sexual selection might be particularly important in influencing which male's sperm are used to fertilize a female's ova when she mates multiply. I examine sperm precedence in oriental beetle in Chapter 4, and show that morphology of the male genitalia—a character that is expected to evolve primarily via post-copulatory sexual selection—is indeed strongly correlated with relative male fertilization success. Despite the fact that post-copulatory sexual selection has long been suggested to be a likely explanation for the complexity and diversity of the genitalia of male insects and other arthropods (Eberhard 1985), mine is one of the few studies to provide empirical evidence supporting this hypothesis.

Interestingly, I also found that characters of the first male to mate had primacy over traits of the second male in impacting relative paternity (Chapter 4). Clarification of the underlying mechanisms (and evolutionary forces behind them) by which certain males achieve greater relative paternity is beyond the scope of my research. However, there is likely strong selection on males to avoid sperm competition by physically preventing other males from copulating with their mate. Likewise, males should be

selected to stimulate ovulation, oviposition, mobilization of sperm, and especially refractory behavior toward additional matings—which could be mediated by transfer of sperm, accessory gland products, and/or copulatory courtship behavior. It is possible that females in which refractory behavior has been strongly stimulated are less likely to use the sperm of any subsequent males that are able to induce copulation. Moreover, if polyandry is relatively uncommon in the field, then selection on means to preempt the sperm of previous mates could be weak. My results represent one of the few cases in insects in which relative paternity is impacted primarily by traits of the first male to mate.

My results in Chapter 4 are also novel in that relative paternity was inversely related to male size. This result is at odds with the general positive relationship between male size and fertilization success in insects (Andersson 1994; Simmons 2001). I hypothesized that larger males may experience greater lifetime reproductive success when given the opportunity to mate multiply; I explore this hypothesis in Chapter 5 by mating males to multiple females. I show that reproductive output of males is reduced in successive matings only for smaller males. These results suggest that after their first mating, smaller males must either compete using a lower quality ejaculate or submit to a longer refractory period to replenish ejaculate reserves. Thus, the sexually selected advantage of smaller males in their first mating is apparently balanced by lower lifetime reproductive potential relative to larger males. I discuss the possibility that males employ different ejaculation strategies based on their size, which may be positively correlated with both ejaculate production as well as mating frequency.

Previous studies with oriental beetle have focused largely on management and monitoring. In addition to investigating mating disruption and working toward an effective and environmentally sound approach to management of oriental beetle, my research demonstrates that numerous fascinating questions regarding the mating behavior, ecology, and evolution of this species remain to be explored.

CHAPTER 2

THE POTENTIAL MANAGEMENT OF ORIENTAL BEETLE IN CRANBERRY BY MATING DISRUPTION USING RETRIEVABLE, POINT-SOURCE DISPENSERS OF SEX PHEROMONE

Abstract

I evaluated the potential for management of oriental beetle in cranberry by disruption of communication between the sexes through application of the female sex pheromone deployed from wax disks. Mean catch of males in pheromone traps on each untreated bog minus that on each pheromone-treated bog was significantly greater than zero throughout most of the male flight period for both years of the experiment. Traps set on the adjacent upland of pheromone-treated bogs showed captures of males that were intermediate between captures on control and treated sites; thus, disruption may be limited just a short distance from pheromone-treated plots, and immigration of beetles from nearby, untreated areas is possible if treatment is not extended to the surrounding upland. To compare mating rates between control and treated sites, I deployed tethered virgin females in the field for two nights in 2003; only females on control sites were mated. In 2004, I measured delayed mating in the field by deploying tethered females within soil-filled pots and estimating mating rates over five nights by the presence or absence of fertile eggs within soil samples. Only one female among the treated sites was mated, while the cumulative percentage of females mated in control sites rose steadily over five nights to nearly 96%. The results of this study provide an empirical test of the importance of delayed mating in field plots subjected to pheromone treatment, and suggest the strong potential of mating disruption for management of

oriental beetle in cranberry using retrievable, high-dose, point-source dispensers of pheromone.

Introduction

Shortly after its introduction in the Northeast U.S. sometime before 1920 (Vittum et al. 1999), oriental beetle, *Anomala orientalis* (Waterhouse) (Coleoptera: Scarabaeidae), became an important turf and ornamental pest. The species has more recently become increasingly problematic in food crops, including blueberry (Polavarapu 1996) and cranberry (Dunn and Averill 1996; Averill and Sylvia 1998). The similar appearance of oriental beetle larvae to those of Japanese beetle (*Popilla japonica* Newman) and the relatively cryptic behavior of adults have contributed to the under-appreciation of oriental beetle's importance as a pest. In mixed populations of the two species in turf, damage is often attributed solely to Japanese beetle (Adams 1949; Vittum et al. 1999). Although adult females may occasionally feed on flowers of various ornamental plants, oriental beetle is not considered a pest in this life stage (Friend 1929; Hallock 1933); the larvae, however, can cause considerable damage to the roots of many plant species. In cranberry, the typically patchy infestations can cause complete local vine dieback within one year and subsequent generations continue to radiate from points of infestation.

Few viable options exist for management of oriental beetle in cranberry. The neo-nicotinoid imidacloprid—the first pesticide available for use against soil insects in cranberry since the ban on the organochlorine insecticides dieldrin and aldrin in the 1970s—has only been registered since 2003, and its efficacy in the field remains

unknown. Not only is imidacloprid expensive in its own right, but its relative ineffectiveness against second and third instars, at least in turf (Potter 1998; Koppenhöfer et al. 2002), means that it must be applied prophylactically to large areas (before damage is evident), which compounds the expense. Imidacloprid also negatively impacts the searching behavior of *Tiphia vernalis* (Hymenoptera: Tiphiidae) (Rogers and Potter 2003), a natural enemy of oriental beetle and Japanese beetle. Moreover, the proximity of many cranberry bogs to residential areas and the close association of bogs with natural watercourses contribute to the importance of establishing more environmentally sensitive approaches to pest management in cranberry.

Recent interest in research on oriental beetle and in pheromone-based management strategies in particular has been generated by the identification of the major components of the female sex pheromone of this species (Leal 1993; Leal et al. 1994; Zhang et al. 1994). Management of oriental beetle by mating disruption shows promise in ornamentals (Polavarapu et al. 2002), blueberry (Polavarapu et al. 2002; Sciarappa et al. 2005), and turf (Koppenhöfer et al. 2005). Polavarapu et al. (2002) used a microdispersible (sprayable) formulation, which (1) showed diminishing efficacy over time, and (2) being a ketone, is restricted from wide scale use on food crops (Weatherston and Minks 1995). Both of these limitations can potentially be overcome by using an alternative formulation for application of the pheromone; ketone pheromones are exempt from restrictions if deployed via retrievable dispensers (Weatherston and Minks 1995), which may maintain more constant release rates over long periods compared with microdispersible formulations (Sanders 1997).

One possible major limitation of mating disruption in general—especially when the target insect occurs on other vegetation surrounding the treated crop—is immigration of the pest from outside the pheromone-treated area (Cardé and Minks 1995). Although dispersal in oriental beetle is limited for both males and females (Facundo et al. 1999a), densities of grubs and adults may be quite high in the upland area surrounding cranberry bogs (personal observation). Therefore, unless disruption of male orientation from pheromone treatment extends to the upland, the possibility exists for re-inoculation of treated bogs with mated females from surrounding areas.

Mating disruption studies typically evaluate efficacy in part by measuring mating rates of virgin females deployed in the field for one night; however, the value of such an assay is limited when females live for more than one night. A plethora of laboratory studies have evaluated delayed mating in insect pests in the lab based on the principle that the effects of mating delay on female reproductive output have important implications regarding the efficacy of mating disruption (see Chapter 3). However, empirical investigations into delayed mating in the field are scarce (but see Knight 1997).

This study was conducted to evaluate the potential use of sex pheromone for mating disruption of oriental beetle in cranberry using pheromone emitted from retrievable wax disks. I compared trap shutdown on treated versus control sites and estimated mating rates using tethered virgin females. Further, because mating disruption treatments may be confounded by immigration of beetles from adjacent, untreated areas, I assessed to what extent males were disrupted in the grassy upland areas surrounding bogs. Finally, in the second year of the study, I deployed tethered

females in the field and estimated mating rates over several nights to evaluate the potential importance of delayed mating in field plots subjected to pheromone treatment.

Methods

Study sites

I conducted all experiments in Massachusetts cranberry bogs with confirmed presence of oriental beetle grubs; I used four pairs of bogs (treated and control) in 2003 and 2004. Every effort was made to match paired sites for infestation level, size, and management regime (i.e. most pairs of sites were managed by the same grower). For each pair of bogs I randomly assigned which one was to be treated, except when close proximity of bogs and prevailing wind direction mandated that the treated bog be downwind of the control. In 2003, two of the pairs of sites were located in Marstons Mills, Barnstable County; one pair was in West Wareham, Plymouth County; and the fourth pair comprised a bog in Marstons Mills and State bog at the University of Massachusetts Cranberry Experiment Station in East Wareham, Plymouth County. Unfortunately, I was unable use all the same field sites when repeating the experiment the following year, primarily due to changing bog ownership. In 2004, one pair was retained from Marstons Mills, and three new pairs of sites were used—one located in Lakeville, Plymouth County; one in Kingston, Plymouth County; and the fourth pair comprised a bog in Plymouth and a bog in Onset, Plymouth County.

Mating disruption

Pheromone on treated bogs was deployed using wax disks. I melted a roughly 500 g block of paraffin wax in a beaker, then added oriental beetle sex pheromone (4.3% by weight; (Z)-7-tetradecen-2-one, Bedoukian Research, Inc., Danbury, CT) and butylated hydroxytoluene (BHT; 8.7% by weight), an antioxidant. In 2004, I omitted the BHT from disks. I poured the molten wax mixture into plastic Petri dishes (inside diameter: 47.8 mm, inside height: 7.6 mm) and allowed the wax to cool slightly before placing in a freezer (-30°C). Each disk weighed ca. 11.5 g and contained ca. 0.5 g of pheromone (and, in 2003, ca. 1 g of BHT). When disks were cool, I popped them out of Petri dishes, drilled a 3.2 mm diameter hole about 1 cm from the edge of each disk, and then attached each disk to a 42 cm long wire stake using a 10.5 cm plastic cable tie.

On 6 July 2003 (9 days after first flight of males) and on 22 June 2004 (6 days after first flight of males), I deployed disks throughout all treated bogs such that they were positioned within the top of the cranberry canopy. Disks were applied at a rate of 100 per ha in a 10 × 10 m grid pattern, resulting in 50 g AI per ha.

On 8-9 July 2003 and on 2-3 June 2004, I set Japanese beetle-style pheromone traps roughly evenly spaced throughout each bog. In 2004, I set the traps well before first flight and before applying pheromone treatment in order to compare pre- and post-treatment captures of beetles. In both years I set traps at a rate of 6 per ha, spaced at least 30 m apart and 20 m from the bog edge; traps were hung from wire stakes with the trap bottom resting on the ground surface. I baited each trap with a 100 µg lure of sex pheromone on a rubber septum, and replaced lures after five weeks in 2003 and after five weeks and ten weeks in 2004. I emptied all traps weekly and counted the number

of beetles captured in each trap; I used the mean number of beetles captured per trap on a bog each week as a single data point. The experiment continued until beetles were no longer collected in traps (24 September 2003 and 8 September 2004).

To estimate trap shutdown, I calculated a disruption index (DI): DI = (C-T)/C × 100, where C = mean number of beetles captured per trap in control plot and T = mean number of beetles captured per trap in treated plot.

In 2004 on 14 July, I set additional pheromone traps on the upland area of treated sites, directly adjacent to the bogs (i.e. not more than 1-2 m from pheromone-emitting wax disks). I placed two traps per site, spaced at least 20 m apart and positioned on the downwind edge of the treated bogs. I emptied these traps weekly and counted the number of beetles captured in each trap; I used the mean number of beetles captured per trap per site each week as a single data point.

Although a putative minor component of the sex pheromone of oriental beetle has been identified, I used only the major component in wax disks and in rubber septa; unlike in many moths (see Minks and Cardé 1988) inclusion of the minor component does not have a significant impact on orientation to synthetic pheromone sources in wind tunnels (Zhang et al. 1994), or pheromone trap captures in the field (Facundo et al. 1994).

Collection and rearing of beetles for tethered virgin female assays

I collected third instar grubs from turf at Norwich Golf Course, Norwich, CT on 15 May 2003 and from Bay Pointe Country Club golf course, Onset, MA on 18 May 2004. All grubs were collected into plastic containers with soil, and then taken back to

the lab where they were placed individually into lidded, clear plastic cups (30 mL) with prepared soil. The soil mixture was made by mixing two parts screened sand with one part screened peat moss (1.18 mm sieve). I sterilized the soil in an autoclave (123˚C, 1 kg/cm^2 for 60 min), allowed it to cool, and then added sterilized, deionized water to moisten the mixture to about 12% water by weight. A heavy pinch of grass seed (Pennington® mix comprised of 63% shining star perennial ryegrass, 15% boreal red fescue, 9% kenblue Kentucky bluegrass, 9% blue bonnet Kentucky bluegrass, 2% inert matter, 1.9% other crop seed, and 0.1% weed seeds) was placed on top of the soil in each cup to provide food for the grubs to continue development. I placed cups on 30-cup capacity clear plastic trays and placed each tray into a clear 9.46-liter plastic, re-sealable bag with a damp sponge to reduce moisture loss from cups. Grubs were held in a rearing chamber on a 16:8 L:D cycle at 10°C to slow development.

About one month before adult beetles were needed, I placed trays in a rearing chamber on a 16:8 L:D cycle at 25°C; adult eclosion began after about 3-4 weeks under these conditions. I checked each cup weekly for pupae, and checked pupae daily for adult eclosion in order to establish adult age. Adults were sexed (based on size of antennal lamellae) and females were held in their rearing cups until needed for deployment in the field.

Tethered virgin female assay, 2003

I compared mating rates on treated and control sites by deploying tethered virgin females in the field. Due to a limited supply of females, only 24 were used in 2003—four females per bog on three of the four pairs of sites. I prepared females on the date

of deployment by tying a 1 m-long 4 lb. test fishing line at the junction of the prothorax and abdomen of females age 4-7 d. On the evening (ca. 1900 h) of 19 July 2003, I placed females in the field inside their un-lidded 30 mL plastic rearing cups with soil; the rearing cup was buried in the ground, with the rim set flush with the soil surface and the tether was tied to a wire stake. Females were placed haphazardly throughout bogs at least 20 m apart and equidistant from four wax disks. I retrieved females at ca. 0600 h on 21 July, thus allowing two evenings for mating to occur; peak male responsiveness to pheromone occurs at dusk (Facundo et al. 1994; E.J.W., unpublished data), which presumably corresponds to peak sexual receptivity in females. I returned females to the lab, cut their tethers, and placed them individually into plastic oviposition cups (height: 13 cm, diameter: 12 cm) with a layer of sterilized soil ca. 3 cm deep. Oviposition cups were held under a 16:8 L:D cycle, and at 25-27°C during photophase and 21-22°C during scotophase and the last two hours of photophase. One week after transfer to oviposition cups, I sorted through soil and collected any eggs into Petri dishes with filter paper moistened with sterilized, deionized water. If the female was still alive, I returned her to the oviposition cup with fresh soil and collected any additional eggs ca. 5 days later. Eggs were held at least until fertility could be assessed; fertile eggs increase in size, become more spherical, maintain their white color, and just before hatch the orange-brown mandibles of the developing larva are visible through the chorion (personal observation). Any female laying fertile eggs was assumed to have mated, while females that laid no eggs or only infertile eggs were assumed to have not mated.

Tethered virgin female assay, 2004

I modified the tethered virgin female assay in order to assess whether delayed mating occurred in the field. I used only females age 4-5 d and left them in the field for five nights, beginning on 23 July; I used 10-12 females for each of the four pairs of sites. I deployed females individually in terra cotta pots (diameter: 10 cm), each lined with a removable, plastic container (volume: 225 mL) filled with sterilized soil; I buried the pots in the bog soil, with the rim set flush with the ground surface. Females were tied to a tether attached to a wire stake (as above), but with the stake inserted through the center of the pot. The tether was kept short enough to restrict females within the confines of the soil in the pot; thus, any mating and subsequent oviposition occurred within the soil provided in the pot. I also modified the tethering technique in 2004. I punctured a hole in the left elytron of each female using a #3 insect pin, then threaded the fishing line through the hole and melted a small portion on the end of the line to prevent it from slipping back through the hole. I collected the soil from each pot after two and three nights, replacing pots with new soil each time. After the fifth night I retrieved all females from the field, along with the last batch of soil. I sorted through each soil sample and maintained any collected eggs to determine fertility and female mating status, as above. Females generally begin laying eggs within 24 h of mating and very rarely lay eggs before mating (E.J.W., unpublished data); thus, the presence or absence of eggs in soil samples was a reliable indicator of when a female mated. After females were returned to the lab, I allowed them to lay any eggs in oviposition cups (see above) for five days before sorting through soil to look for any eggs. Some mortality

(apparently from birds) occurred in the field, especially on control plots; I replaced any missing females the same day they were found to be missing.

Data analysis

In order to control for unequal variance between treatments (see error bars in Figures 2.1-2.2), I calculated the difference between the mean number of beetles captured on each control site and those captured on each paired treated site. I then log (Y + 1) transformed these difference values to achieve normality. To compare pheromone trap captures between traps on the upland of treated bogs versus those on control and on treated sites, I handled the data similarly, except that values were fourth root-transformed for [control – upland] captures and square root-transformed for [upland – treated] captures. I tested for normality by Kolmogorov-Smirnov test on the residuals derived from preliminary analyses using untransformed data. For each week, I separately ran one-sample t-tests to compare the mean difference for a given contrast with zero ($\alpha = 0.05$). All data were analyzed using SAS (version 8.2, SAS Institute, Inc.).

Results

Mating disruption, 2003

Mean beetle captures were consistently higher on control sites than on the paired treated sites (Figure 2.1). The difference in pheromone trap captures between bogs treated with oriental beetle sex pheromone and control sites was significantly greater than zero for all but the last two weeks of the experiment (Table 2.1; Figure 2.1). The

mean disruption index for all sites across the ten weeks of the study in 2003 was 92.2% and ranged from about 80 to 99% (Table 2.3).

Tethered virgin female assay, 2003

Of the 24 virgin females deployed across six of the eight bogs, only three were mated—one female from each of the three untreated bogs.

Mating disruption, 2004

Again, mean beetle captures were consistently higher on control sites than on the paired treated sites (Figure 2.2). The difference in pheromone trap captures between pairs of sites was not significantly different from zero for the first two weeks of the experiment, before pheromone was applied to the treated sites (Table 2.2; Figure 2.2). One week following treatment, captures of beetles in pheromone traps tended to be lower for the pheromone-treated sites (Figure 2.2), but the difference was not significant (Table 2.2). For the remaining nine weeks of the experiment, the difference in trap captures between pheromone-treated sites and untreated controls was significantly greater than zero (Table 2.2; Figure 2.2). The mean disruption index for all sites across weeks 3-12 was 92.3% and ranged from about 80 to 98% (Table 2.3).

Beetle captures in pheromone traps set on the upland of treated bogs were generally intermediate between the number collected from the adjacent treated bogs and paired control bogs (Figure 2.2). For nearly every week that upland pheromone traps were deployed, mean beetle captures in these traps were significantly lower than were

captures on paired control sites, but significantly higher than on the adjacent treated bogs (based on difference contrasts; Table 2.4).

Tethered virgin female assay, 2004

I observed a clear difference in mating rates between females deployed on pheromone-treated sites versus those on control sites (Figure 2.3). After two nights in the field, a mean of 64% of females had been mated on control bogs. The cumulative portion of females that were mated rose steadily and, after five nights, nearly 96% of females on control sites had been mated. In contrast, only one female was mated on the pheromone-treated bogs; she was mated within the first two nights of deployment (Figure 2.3).

Discussion

The results reported here demonstrate the strong potential of mating disruption for management of oriental beetle in cranberry. Mating rates of tethered virgin females were markedly reduced in pheromone-treated bogs, indicating that mating was indeed disrupted. Captures of male beetles were consistently higher on control sites than on paired treated sites over the course of both years of the experiment. Only in the last two weeks of male flight in 2003 were differences in pheromone trap captures not significant; by this time in the season, the number of beetles in flight may have been biologically unimportant. In 2004, the difference in trap captures between control and treated sites was significant through the end of the flight season. Polavarapu et al. (2002) found evidence of diminished effectiveness over time for mating disruption of

oriental beetle using a sprayable formulation; however, my results suggest that the use of a high-dose, point-source formulation can effectively disrupt mating for the duration of the season. Sciarappa et al. (2005) showed strong trap shutdown of oriental beetle in blueberry using a similar point-source formulation, but it is unclear from their data whether there was a reduction in effectiveness over time.

Unfortunately, patchiness of larval distributions and the destructive nature of sampling grubs in cranberry beds precluded an assessment of the effects of pheromone treatment on the subsequent generation of beetles within bogs. Nevertheless, I observed striking differences in mating rates on control versus treated sites. Female oriental beetles may live in the lab for roughly two weeks and do not exhibit a strong decline in reproductive output until about 5-6 d after reaching sexual maturity (see Chapter 3), so the possibility exists for females to experience delayed mating in pheromone-treated plots, but still realize a high reproductive output. Despite the relative resilience of females to mating delay in the lab (Chapter 3), my results here suggest that—even when deployed in the field over five nights—the likelihood of females mating on pheromone-treated plots is small. Most studies of mating disruption that use tethered females to estimate mating rates only deploy females in the field for one night; considering that insect pests are generally reproductively mature for more than one night, the utility of such an assay is clearly limited. Thus, my results are particularly valuable and suggest that pheromone treatment may not merely delay, but *prevents* mating for a large enough portion of the females in a local population to make delayed mating an unimportant force in management of oriental beetle.

Calling females in pheromone-treated plots may be exposed to potential predators for far longer than they otherwise would be if mated within the first few nights of calling, so predation could conceivably contribute to management by mating disruption. However, most of the predation that I observed for tethered females occurred in the control plots, apparently by birds that were attracted to the vicinity of tethered females by the enhanced local activity of males (personal observation). I observed no such male activity in pheromone-treated plots; therefore, predation by birds would probably contribute little to a mating disruption program. At least one female was apparently preyed upon by an arthropod with piercing/sucking mouthparts, so generalist, cursorial arthropod predators (e.g., spiders) might be a small factor in mortality of calling females in pheromone-treated sites.

Although in both years of the study the mean disruption index (DI) among all sites was >90%, it ranged from about 80 to 99% (Table 2.3). The sites with lower DI apparently did not exhibit lower disruption due to particularly high population densities, at least based on captures in paired control plots. The one site that showed a low DI in 2003 was comprised of two distant pairs of bogs that may have been poorly matched for infestation level. For site 3 in 2004, the DI was 80% over weeks 3-12 of the study (Table 2.3), but 93% for weeks 4-12; thus, this site exhibited relatively low disruption in the early weeks of the experiment and higher disruption later. It is possible that differences in ambient temperature and/or wind currents affected the rate at which pheromone from wax disks was released and dispersed across the bogs. In 2004, I waited to deploy disks in order to compare pre- and post-treatment pheromone trap captures; in practice, however, given that oriental beetle is protandrous (Facundo et al.

1999b), establishing mating disruption treatments immediately following first flight of males could ensure effective disruption of mating by the time of female emergence.

Although orientation to pheromone sources is apparently inhibited to some extent in the upland of treated sites, disruption might not be strong enough to impact the upland populations of beetles. Indeed, a potential major limitation of mating disruption of oriental beetle is the wide host range of the larvae, which means that treated areas could be re-inoculated by gravid females from nearby untreated areas. Turf surrounding cranberry may harbor greater densities of grubs and adults than the bogs themselves (E.J.W., unpublished data), so immigration of mated females onto bogs seems possible and is likely the source from which infestations on cranberry originate. Although the restricted dispersal tendencies of oriental beetle (Facundo et al. 1999a; see below) may limit potential problems with immigration of beetles onto treated plots, extending pheromone treatment to the surrounding upland could be a means to further reduce possible immigration issues.

Recent work on mating disruption of oriental beetle in blueberry demonstrates the possibility for >95% trap shutdown with nearly an order of magnitude lower AI than was used in my study (Sciarappa et al. 2005). Although mean capture of males at peak flight was 4-5 times higher in my study than in the Sciarappa et al. (2005) work in blueberry, it would be worth investigating the minimum application rate necessary to effectively disrupt mating in cranberry. Synthetic sex pheromone of oriental beetle is expensive and application may be labor intensive compared to spraying conventional pesticides; lowering the necessary application rate could have a significant impact on the overall cost of mating disruption to growers.

Mating disruption remains probably the most widespread application of semiochemicals in insect pest management; however, the technique has been limited almost exclusively to moths. Work with other insects [e.g., scolytine bark beetles, Coleoptera: Curculionidae (Trudel et al. 2004); plant bugs, Heteroptera: Miridae (Kakizaki 2004); and sawflies, Hymenoptera: Diprionidae (Östrand et al. 1999)] suggests that mating disruption has strong potential beyond the Lepidoptera. The results presented here and elsewhere for oriental beetle (Polavarapu et al. 2002; Sciarappa et al. 2005) support this assertion. There is nothing apparent about the biology of moths that lends this group to management by semiochemicals more than other orders. In fact, several aspects of oriental beetle biology contribute to the promise in this species for effective management by mating disruption.

First, feeding by adults is rare and virtually nonexistent in males (Friend 1929; Hallock 1933), likely making the influence of pheromones on male behavior more important than in species that may spend a considerable amount of time responding to plant kairomones and feeding (Hasegawa et al. 1993). Second, although females only call for a few hours in the evening (Facundo et al. 1999a), males are at least somewhat responsive to pheromone 24 hours per day (Facundo et al. 1994); therefore, it might be possible to physically and/or sensorially fatigue males for long periods with little likelihood of males chancing upon calling females. It should be noted that saturation of crops with synthetic sex pheromone could potentially increase or decrease male searching behavior (Sanders 1997), but I caution that I have no empirical data on the dispersal behavior of male oriental beetle in field plots subjected to mating disruption treatment. Third, because the major pheromone component alone is sufficient to elicit

successful orientation behavior of males in the lab (Zhang et al. 1994) and to maximize pheromone trap captures in the field (Facundo et al. 1994), effective disruption may be more economical than in other species that require a two- or more component blend. However, a potential long term drawback of using only the major component of the female sex pheromone for mating disruption is the possibility of the development of resistance mediated by selection on males to respond more strongly to minor components of the sex pheromone and selection on females to adjust their production of the pheromone blend to increase the relative portion of the minor component. Fourth, most males may be able to mate at most only once per evening (see Chapter 5); thus, the ability of a few males to fertilize a large number of females is probably relatively restricted compared to some species of moths in which males may mate four to five times per night (e.g., Amoako-Atta and Mills 1977; Unnithan and Paye 1990, 1991; Jiménez-Pérez and Wang 2004a). Finally, dispersal is limited for both males and females (Facundo et al. 1999a), so the possibility of immigration of males or, worse yet, mated females onto a site undergoing mating disruption will likely be small. Female dispersal might only be associated with the seeking of floral feeding sites following a first bout of egg laying (Facundo 1997). Although it is not known whether females feed on the flowers of cranberries, the period of cranberry flowering in Massachusetts generally ends at least one week before activity of adult oriental beetles peaks; therefore, immigration of mated females in search of flowers to feed upon should be minimal.

The data presented here demonstrate the potential for using retrievable, high-dose, point-source dispensers of the major component of female sex pheromone for

mating disruption of oriental beetle in cranberry. Despite the encouraging results shown here, the effectiveness of disruption of male orientation to pheromone traps diminishes somewhat just a short distance from treated areas. Also, the high cost of oriental beetle sex pheromone and the labor necessary to prepare, install, and remove dispensers may make mating disruption an impractical alternative to traditional pesticides at present. Future work should be aimed at determining the minimum effective application rate in cranberry, in conjunction with assessing the value of treating both the target crop and the surrounding vegetation, which may harbor large numbers of beetles. Labor costs may be reduced by developing more simple, easily prepared pheromone dispensers. Reducing the costs associated with mating disruption of oriental beetle in cranberry may make this effective and environmentally sensitive management approach also economically feasible.

Table 2.1. Summary of differences (mean ± SE) in pheromone trap captures in treated versus control sites across each week in 2003. *Difference* column represents mean ± SE (untransformed) of number of beetles captured in control bogs minus number captured in paired treated bogs. For each week, t-tests compare log-transformed difference values with zero.

Date in 2003	Difference	t-value	P-value
16 July	370.3 ± 182.0	5.2	0.036
23 July	327.0 ± 194.2	7.2	0.006
30 July	385.3 ± 223.7	7.3	0.005
6 August	171.4 ± 120.3	6.7	0.007
13 August	183.6 ± 121.4	7.6	0.005
20 August	16.2 ± 5.7	9.6	0.002
27 August	3.93 ± 0.72	9.1	0.003
3 September	0.34 ± 0.08	4.7	0.018
10 September	0.28 ± 0.19	1.6	0.206
17 September	-0.03 ± 0.09	-0.45	0.682

For 16 July 2003, n = 3; n = 4 for remaining weeks. Treatment was initiated on 6 July.

Table 2.2. Summary of differences (mean ± SE) in pheromone trap captures in treated versus control sites across each week in 2004. Difference column represents mean ± SE (untransformed) of number of beetles captured in control bogs minus number captured in paired treated bogs. For each week, t-tests compare log-transformed difference values with zero.

Date in 2004	Difference	t-value	P-value
16 June	1.04 ± 1.13	2.7	0.228
22 June	0.83 ± 1.20	-0.39	0.724
30 June	40.3 ± 32.5	2.0	0.142
7 July	233.4 ± 74.9	14.2	0.001
14 July	166.1 ± 44.5	16.9	0.001
21 July	462.8 ± 146.6	20.1	0.0003
28 July	159.8 ± 73.0	11.8	0.001
4 August	108.8 ± 45.5	11.8	0.001
10 August	23.1 ± 11.2	5.0	0.015
17 August	8.0 ± 5.6	4.1	0.026
25 August	4.4 ± 3.1	3.6	0.036
1 September	1.4 ± 0.18	23.2	0.002

For 16 June 2004 n = 2; for 1 September 2004 n = 3; n = 4 for all other weeks. Treatment was initiated on 22 June.

Table 2.3. Mean ± SE beetles per site per week and mean ± SE disruption index per week for each pair of sites over the course of both years of the study.

		2003		2004	
Site	Treatment	Beetles per trap	DI	Beetles per trap	DI
1	control	92.8 ± 14.3	95.8 ± 0.5	97.1 ± 38.5	97.3 ± 0.7
	treated	3.9 ± 0.5	—	2.8 ± 0.9	—
2	control	82.3 ± 12.3	93.7 ± 1.5	77.6 ± 32.5	94.6 ± 1.5
	treated	5.2 ± 0.8	—	2.2 ± 0.8	—
3	control	371.7 ± 41.2	99.0 ± 1.4	68.2 ± 35.6	79.5 ± 13.6
	treated	3.8 ± 0.5	—	5.1 ± 2.0	—
4	control	17.2 ± 1.9	80.4 ± 1.2	203.1 ± 81.2	97.7 ± 1.3
	treated	3.4 ± 0.5	—	2.0 ± 0.6	—

Disruption index in 2004 was calculated for weeks 3-12 (i.e. after pheromone treatment was initiated). Site 2 was identical in both years, but the remaining sites differed between years.

Table 2.4. Summary of differences (mean ± SE) in pheromone trap captures on control sites versus upland of treated sites and treated sites versus upland of treated sites for each week in 2004. *Difference* columns represent mean ± SE (untransformed) of the contrasts shown. For each week t-tests compare fourth root-transformed difference values (for [control – upland]) or square root-transformed difference values (for [upland – treated]) with zero.

Date in 2004	Difference: [control - upland]	t-value	P-value	Difference: [upland - treated]	t-value	P-value
21 July	329.7 ± 158.5	8.9	0.003	130.9 ± 50.70	5.5	0.012
28 July	122.1 ± 69.40	6.7	0.007	38.5 ± 3.60	21.90	00.0002
04 August	73.1 ± 44.3	5.8	0.010	36.0 ± 6.60	10.70	0.002
10 August	17.9 ± 10.8	5.9	0.028	5.6 ± 1.4	8.6	0.003
17 August	6.0 ± 6.0	4.3	0.145	2.0 ± 1.5	6.3	0.024
25 August	3.3 ± 2.5	5.7	0.029	1.1 ± 0.7	4.3	0.051
01 September	0.9 ± 0.4	5.7	0.029	0.8 ± 0.6	1.7	0.225

For 1 September 2004, n = 3; n = 4 for all other weeks.

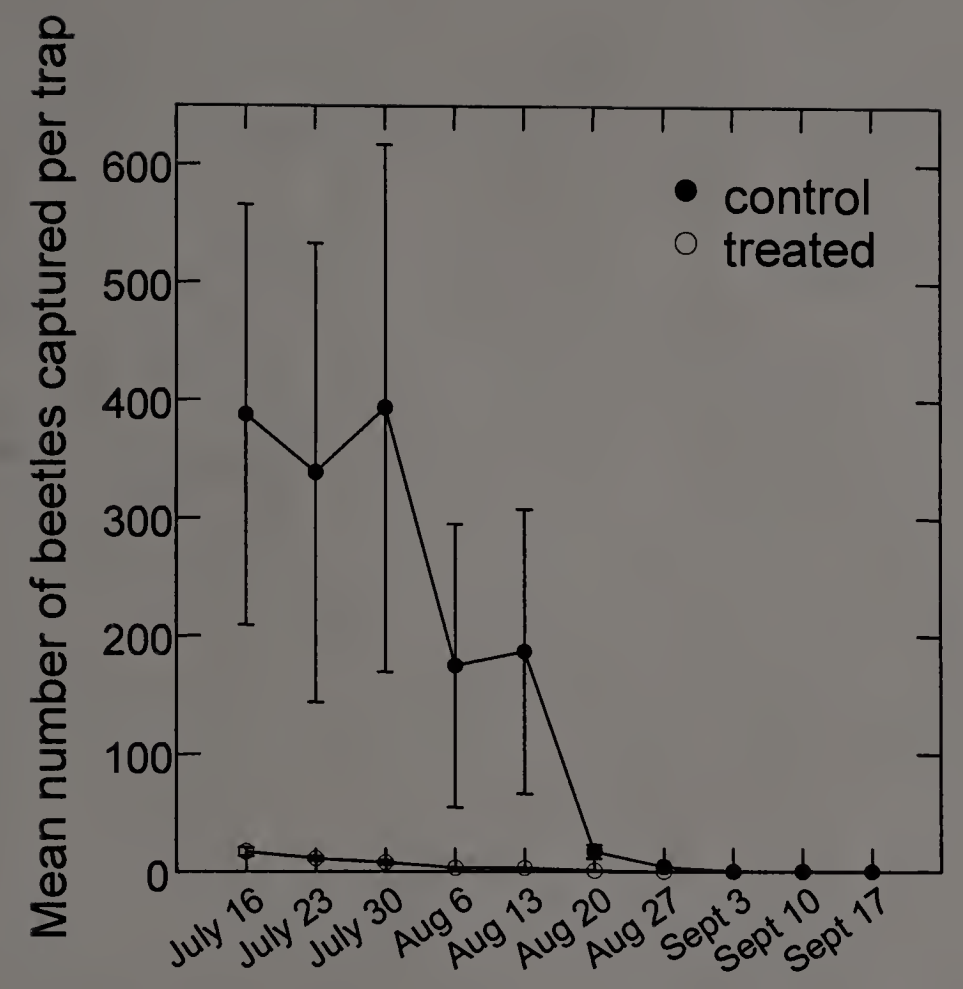


Figure 2.1. Captures of male oriental beetle in pheromone traps set in cranberry bogs in 2003. Pheromone treatment was initiated on 6 July. Error bars represent standard error.

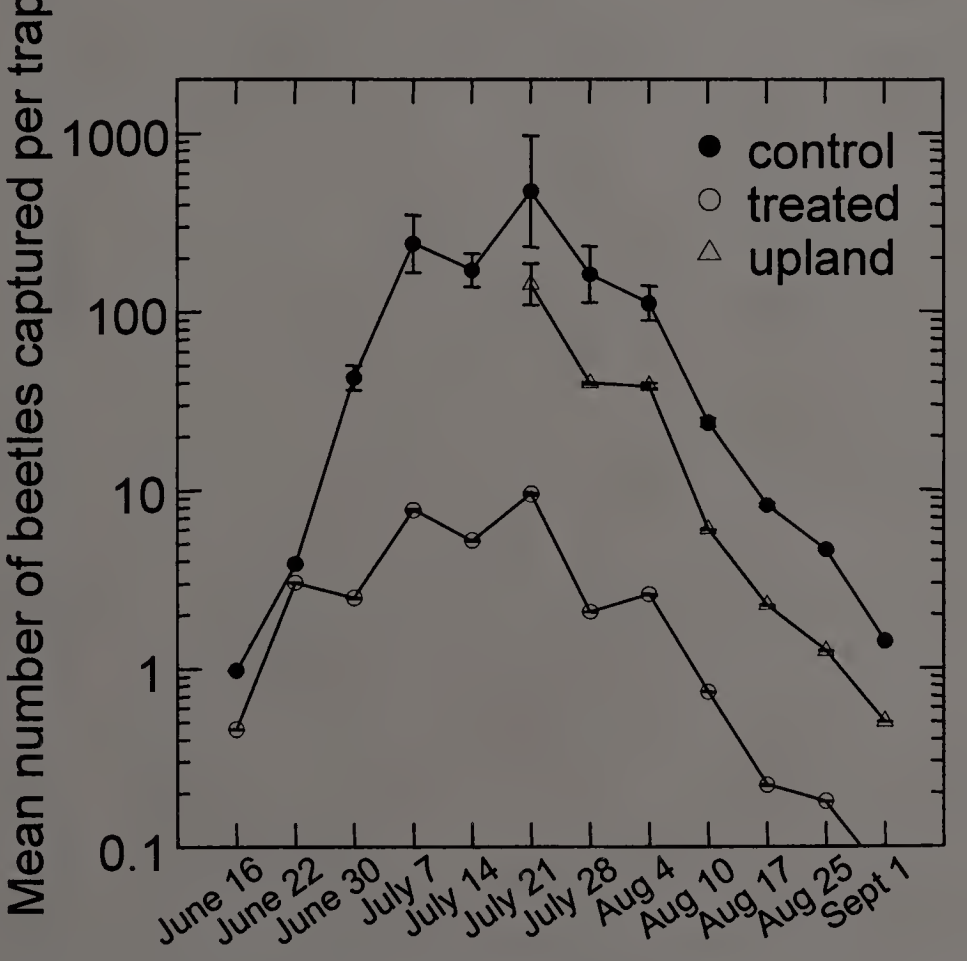


Figure 2.2. Captures of male oriental beetle in pheromone traps set in cranberry bogs in 2004. Upland traps were set directly adjacent to pheromone-treated plots in the grassy areas surrounding the bogs. Pheromone treatment was initiated on 22 June. Note logarithmic y-axis; error bars represent standard error.

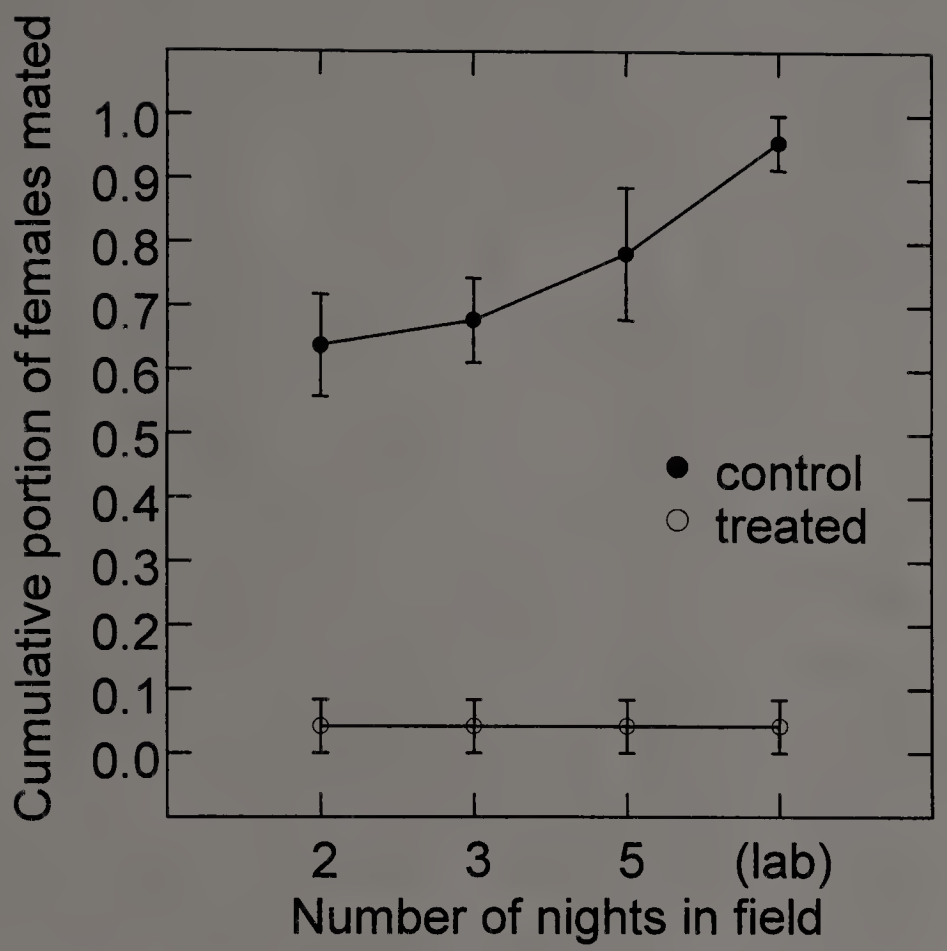


Figure 2.3. Cumulative portion of tethered virgin females placed in the field that were mated, based on presence or absence of fertile eggs in soil samples. Females were left in the field for five nights, then taken back to the lab where they were allowed to lay eggs if they had not yet done so in the field. Error bars represent standard error.

CHAPTER 3

EFFECTS OF DELAYED MATING ON REPRODUCTIVE OUTPUT OF FEMALE ORIENTAL BEETLE

Abstract

I studied the effects of delayed mating on several aspects of the oviposition behavior and biology of oriental beetle using females mated in the lab at 4-13 d post-eclosion. I assessed daily egg output for each female and evaluated how age at mating affected: total fecundity, number of eggs laid per day, egg fertility, female fertility, lifespan, duration of oviposition and pre-oviposition periods, and number of mature eggs remaining in the abdomen at death. Oriental beetle was relatively resilient to the effects of delayed mating. Females exhibited a gradual decline in fecundity with increased age at mating that was largely a function of a decline in duration of the oviposition period as well as lower female fertility: females mated at $\geq$ 11 d were less likely to lay any fertile eggs. Egg fertility, however, did not vary with female age at mating. Because mating delay did not affect longevity, females mated at older ages experienced decreased oviposition periods; however, females laid more eggs per day with increased age at mating, which partially compensated for the smaller window of time in which to lay eggs. A mating delay of six or more days relative to females mated within the first day of reaching sexual maturity resulted in ca. 35-50% lower mean fecundity. These results suggest that in order for mating disruption to be a successful management tool for oriental beetle, mating must be prevented rather than delayed. I compare my study with the vast literature on delayed mating in moths and discuss the

importance of these results in relation to management of oriental beetle using female sex pheromone.

Introduction

Oriental beetle, *Anomala orientalis* (Waterhouse) (Coleoptera: Scarabaeidae), has become a problematic pest of turfgrass and ornamentals in the Northeast U.S. since its introduction sometime before 1920 and has more recently been reported in crops such as strawberry, blueberry (Polavarapu 1996), and cranberry (Dunn and Averill 1996; Averill and Sylvia 1998). Adult females may occasionally feed on flowers of various ornamental plants, but oriental beetle is not considered a pest in this life stage (Friend 1929; Hallock 1933); however, feeding by the larvae can cause considerable damage to the roots of many plant species. The importance of oriental beetle as a pest has been under-appreciated, probably because adults are relatively inconspicuous, and the larvae are difficult to distinguish from Japanese beetle (*Popilla japonica* Newman) larvae without magnification. In mixed populations of the two species in turf, damage is often attributed solely to Japanese beetle (Adams 1949; Vittum et al. 1999).

The identification of the female sex pheromone of oriental beetle (Leal 1993; Leal et al. 1994; Zhang et al. 1994) has encouraged recent interest in this species, and has made possible research into the development of mating disruption in ornamentals (Polavarapu et al. 2002), blueberry (Polavarapu et al. 2002; Sciarappa et al. 2005), turf (Koppenhöfer et al. 2005), and cranberry (Chapter 2). Mating disruption of insect pests by interference of pheromonal communication between the sexes relies on saturation of a target area with synthetic sex pheromone in order to negatively impact male

orientation to calling females. Ideally mating is prevented, but inhibiting the orientation ability of males might only result in a delay of mating for some females, in part because some males may find females by chance, if given enough time. However, even if a portion of the females within a population mate eventually, management by mating disruption may still be effective if females mated at older ages exhibit an appreciable reduction in the number of viable offspring that they produce.

Numerous studies with different species of Lepidoptera—the group that has received the most attention regarding pheromone-based management—have demonstrated reduced reproductive output of females with increased age at mating (see Table 3.1). Further, mathematical models demonstrate that—by reducing the total fertile egg output per female—delayed mating may make an important contribution to the effectiveness of mating disruption in reducing the total size of a pest population (Kiritani and Kanoh 1984; Barclay and Judd 1995; Jones and Aihara-Sasaki 2001). Although these models have yet to be tested with data from the field, it is generally regarded that a very strong impact on female reproductive output is required in order to make delayed mating a significant force in mating disruption. For example, assuming females of a given species live for 10 days, a 90% inhibition of mating per day would still result in up to 65% of females eventually mating ($1 - 0.9^{10} = 0.65$; Kiritani and Kanoh 1984). Depending upon how female age at mating impacts reproductive output, delayed mating has the potential to exert a significant influence on the efficacy of mating disruption beyond the effect of any reduction in mating rates.

Unfortunately, studies of delayed mating in the field are scarce (but see Knight 1997 and Chapter 2); however, laboratory studies remain an important first step toward

assessing the potential importance of delayed mating in pheromone-based management, especially since—despite the nearly universal trend of decreased reproductive output with increasing female age at mating—the degree of impact of delayed mating can vary considerably among species (see Table 3.1 and references therein). In order to develop effective pheromonal control strategies, it is important to have a thorough understanding of the reproductive biology and behavior of the target species, particularly the mating and egg-laying capacities and the factors that influence them (Unnithan and Paye 1990).

This study was designed to evaluate the impact of delayed mating on the reproductive output of female oriental beetle. Pheromone-based management is limited almost exclusively to the Lepidoptera, and little work has been done on delayed mating in other orders. To my knowledge, this study represents the first report of the effects of delayed mating on female reproductive output in a coleopteran species.

Methods

Collection and rearing of beetles

I collected third instar grubs from turf at Norwich Golf Course, Norwich, CT on 15 May 2003 and from cranberry in Marstons Mills, MA on 5 June 2003. All grubs were collected into plastic containers with soil, and then taken back to the lab where they were placed individually into lidded, clear plastic cups (30 mL) with prepared soil. The soil mixture was made by mixing two parts screened sand with one part screened peat moss (1.18 mm sieve). I sterilized the soil in an autoclave (123˚C, 1 kg/cm^2 for 60 min), allowed it to cool, and then added sterilized, deionized water to moisten the mixture to about 12% water by weight. A heavy pinch of grass seed (Pennington® mix

comprised of 63% shining star perennial ryegrass, 15% boreal red fescue, 9% kenblue Kentucky bluegrass, 9% blue bonnet Kentucky bluegrass, 2% inert matter, 1.9% other crop seed, and 0.1% weed seeds) was placed on top of the soil in each cup to provide food for the grubs to continue development. I placed cups on 30-cup capacity clear plastic trays and placed each tray into a clear 9.46-liter plastic, re-sealable bag with a damp sponge to reduce moisture loss from cups. Grubs were held in a rearing chamber on a 16:8 L:D cycle at 10°C to slow development.

About one month before adult beetles were needed, I placed trays in a rearing chamber on a 16:8 L:D cycle at 25°C; adult eclosion began after about 3-4 weeks under these conditions. I checked each cup weekly for pupae, and checked pupae daily for adult eclosion in order to establish adult age. Adults were sexed (based on size of antennal lamellae) and held in their rearing cups until needed for mating experiments.

Mating experiments and oviposition assessment

I mated beetles in the lab under conditions that simulated dusk—the period of peak female sexual receptivity (Facundo et al. 1994) and peak male responsiveness to pheromone (Facundo et al. 1994; E.J.W., unpublished data). I moved beetles to a room held at about 60% humidity and maintained on a 16:8 L:D cycle (as above for rearing chambers), but at 25-27°C during photophase and 21-22°C during scotophase and the last two hours of photophase. Light intensity during photophase was held at ca. 150 lux, except during the first and last two hours of photophase when it was held at 15 lux. I paired beetles within the last two hours of photophase.

I held virgin females for 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 d after eclosion before pairing with 4- to 11-day-old virgin males; laboratory-reared females reach sexual maturity by age 4-5 d (Facundo et al. 1999a; E.J.W., unpublished data) and males age ≥ 4 d display peak response to female sex pheromone (Zhang et al. 1994). I used 5-7 females for each age treatment (see Table 3.3 for precise sample sizes). For each pairing, I placed a female in a plastic, hexagonal balance tray (bottom area: 20.4 cm^2) with a layer of soil ca. 1 cm deep. Females typically began calling (i.e. raising the abdomen and releasing sex pheromone) within 2 min of being placed on soil under simulated dusk conditions (above). I introduced a male after the female began calling; males promptly mounted calling females.

After each pair separated, I transferred the female to a clear, plastic oviposition cup (diameter: 12 cm, height: 13 cm) with a layer of soil ca. 3 cm deep. The soil used for mating and oviposition substrate was identical to that used for rearing (see above). About 36 h after mating and every 24 h thereafter (at ca. 0730h) until her death, I replaced the soil in each oviposition cup and collected any eggs from the previous day's soil. I counted the number of eggs each female laid and transferred the eggs to Petri dishes with filter paper moistened with sterilized, deionized water. Eggs were held at least until fertility could be assessed; fertile eggs increase in size, become more spherical, maintain their white color, and just before hatch the orange-brown mandibles of the developing larva are visible through the chorion (personal observation). After each female died, I dissected her and counted the unlaid eggs remaining in her abdomen; I also measured the length of her right elytron using an ocular micrometer.

In addition to using females of different ages, I also used a virgin female treatment and a treatment designed to test for the possible negative impact of daily handling on female egg production ("clutch treatment"). For the virgin treatment, I placed 8-d-old unmated females in oviposition cups and evaluated egg-laying as above; for the clutch treatment I mated 5-d-old females and transferred them to oviposition cups and replaced soil only when the female had burrowed up to the soil surface.

Data analysis

In nearly all cases, data were normally distributed (based on Kolmogorov-Smirnov test); otherwise I relied on the robustness of linear regression to slight deviations from normality with a large sample size. Rather than transforming any data that did not meet the assumption of equal variance, I used weighted least squares regression. For any data with severe deviations from normality that could not be addressed by transformation, I used nonparametric statistics. When necessary, weights were calculated using the absolute value of each residual ($|e_i|$) from ordinary least squares regression as an estimate of standard deviation (σ_i); each observation was then weighted by the reciprocal of the estimate of its variance ($1/\sigma_i^2$). All data were analyzed using SAS (version 8.2, SAS Institute, Inc.).

Using backwards multiple regression with female age at mating and female size (as estimated by right elytron length) as the main effects, I modeled each of the following variables: total number of eggs laid, number of eggs laid per day, duration of adult female lifespan, number of eggs remaining in abdomen at death, duration of the oviposition period (time in d that spanned the dates when the first and last eggs were

laid), and total egg production (number of eggs laid + number of eggs remaining in the abdomen at death). I began with a multiple regression model that included five parameters: female age at mating (linear and quadratic), female size (linear and quadratic), and the interaction between the linear terms. One by one, I manually removed each term from the model that was not statistically significant ($\alpha = 0.05$) or nearly so ($\alpha = 0.10$); however, female age at mating (linear) was retained in every model, even if its effects were not statistically significant. Terms were dropped from the model if they were statistically significant, but spuriously so (i.e. when the data fit a pattern that represented the base of a very wide parabola, which was essentially a reflection of a zero-slope fit to the data). The independent variables were centered to reduce problems with collinearity.

I used Kruskal-Wallis tests to compare egg fertility (percentage of eggs laid that were fertile) and duration of the pre-oviposition period (time in d between mating and laying first batch of eggs) among age treatments. I also calculated female fertility (percentage of females laying fertile eggs) for each treatment and compared among treatments using Fisher's exact test.

When comparing unmated females with those that were mated at different ages, I performed one-way ANCOVAs with female elytron length as the covariate variable. When ANCOVA showed significant differences for a response variable, I used Dunnett's tests to determine whether each treatment differed from the unmated female treatment ($\alpha = 0.05$). I used female size as estimated by right elytron length in analyses as a dependent variable (for linear regression) or as a covariate (for ANCOVA) for three reasons. First, my previous data using field-collected females showed that among seven

morphological measures, elytron length was the best correlate of total dry mass (r^2 = 0.660, t = 8.8, P <0.0001, n = 42) and, therefore, was a good metric of female size. Also, in this study I found a positive correlation between female elytron length and my estimate of total eggs produced (number of eggs laid + number of eggs remaining in the abdomen at death; r^2 = 0.308, t = 4.9, P <0.0001, n = 65). Finally, in order to count the number of eggs remaining in each female's abdomen after death, destructive sampling was necessary; however, measuring female elytron length was still easily accomplished after dissecting females.

Results

Neither fecundity (t-test: t_{10} = 1.3, P = 0.232) nor egg fertility (Wilcoxon signed rank test: S_9 = 30.0, P = 1.00) differed between clutch treatment females and females mated at age 5 d. Thus, the handling of females to assess daily egg output was deemed not to have a significant impact on oviposition behavior, and clutch treatment females were excluded from subsequent analyses.

The total number of eggs laid by females was dependent upon female age at mating (linear and quadratic) as well as female size (Table 3.2-A; Figure 3.1). Fecundity showed a linear and curvilinear decline with increased delay in mating; thus, the number of eggs laid was negatively correlated with mating delay, but only in females older than 9 or 10 days was mean fecundity markedly reduced (Figure 3.1-A). Fecundity was positively correlated with female size (Table 3.2-A; Figure 3.1-B).

Egg fertility averaged ca. 90% across treatments and did not differ by age at mating (Table 3.3); female fertility, however, differed across age treatments (Table 3.3).

In particular, female fertility tended to be lower for the three oldest age classes; only one female of those mated at age 4-10 d did not lay fertile eggs, while seven of the fifteen females mated at age 11-13 d were infertile (Table 3.3).

Total egg production was positively correlated with female size (Table 3.2-B; Figure 3.2-B), but was not correlated with mating age (Table 3.2-B; Figure 3.2-A). The number of eggs produced by females did not differ among unmated females and those mated at different ages (Table 3.4).

The number of eggs remaining in the female abdomen at death increased linearly and curvilinearly with mating delay (Table 3.2-C; Figure 3.3-A), but was unrelated to female size (Figure 3.3-B). The number of eggs remaining in the abdomen at death differed significantly among females mated at different ages and unmated females (Table 3.4); in particular, the number of unlaid eggs did not differ between unmated females and those mated at 12 and 13 d, but did differ significantly between unmated females and each of the remaining age treatments.

The number of eggs laid per day by females was positively correlated with increased age at mating (Table 3.2-D; Figure 3.4-A), but was unrelated to female size (Figure 3.4-B). Thus, even though larger females produced more eggs, they did not exhibit a higher rate of oviposition.

Adult female lifespan averaged 15.7 d across treatments (including unmated females) and was unrelated to mating age (Table 3.2-E; Figure 3.5-A) or mating status (that is, unmated females did not show an increased lifespan relative to those that mated; Table 3.4). The effect of female size on lifespan was marginally significant [P =

0.0508, 95% confidence interval = (-0.0008, 0.434); Table 3.2-E; Figure 3.5-B], but the full model including mating age and female size was not significant (Table 3.2-E).

Duration of the pre-oviposition period did not differ across age treatments (Table 3.3); however, oviposition period declined linearly with increased delay of mating (Table 3.2-F; Figure 3.6-A). Duration of oviposition period was not correlated with female size (Figure 3.6-B).

Discussion

Fecundity, egg fertility, and female fertility

Overall, oriental beetle appears to be relatively resilient to the negative effects of delayed mating. Fecundity declined with increasing female age at mating, but only to a limited extent. The curvilinear relationship between fecundity and female age at mating reflected a threshold effect wherein fecundity was notably reduced only after a six or more day delay in mating (i.e. for females mated at $\geq$ 10 d post-eclosion). Females mated at 11-13 d laid on average ca. 35-51% fewer eggs than those mated at 5 d. In contrast, a 40-50% reduction in fecundity with just a 2-3 d delay in mating is not uncommon in the Lepidoptera (e.g., Rojas and Cibrian-Tovar 1994; Karalius and Būda 1995; Leather et al. 1985; Proshold 1996; Knight 1997). Moths also often exhibit a more linear decline in fecundity with mating delay, rather than the type of threshold effect that I observed with oriental beetle.

Also unlike in most moths that have been studied, egg fertility of oriental beetle did not decrease with mating delay; females fertilized the same portion of their eggs regardless of the age at which they mated. However, female fertility was lower for the

older age classes, again reflecting a threshold effect wherein females mated at 11-13 d showed a dramatic drop in the likelihood of laying fertile eggs. In fact, reduced fecundity of females mated at older ages was apparently a reflection of both lower female fertility as well as a general tendency to lay fewer eggs. Unfortunately, changes in female fertility for moths with increased age at mating are rarely reported (females that fail to lay eggs may even be excluded from analyses), so a general trend regarding the effect of mating delay on female fertility has yet to emerge. However, at least three studies have reported a decline in female fertility with increased age at mating (Walker 1991; Fraser and Trimble 2001; Torres-Vila et al. 2002), and Huang and Subramanyam (2003) found that female Indianmeal moths were less likely to receive a spermatophore when mated later in life. I did not check for successful sperm transfer in my study, but neither copula duration ($r^2 = 0.003$, $t = -0.37$, $P = 0.714$) nor male copulatory behavior (personal observation) were related with female age at mating, suggesting that males do not assess female age and change their mating behavior accordingly.

Among the many moths that have been studied to date, a reduction in fecundity with increased female age at mating is a nearly universal pattern (Table 3.1). In almost all of these studies, egg fertility also declined with increased female age at mating. I am aware of only three cases in moths in which a decrease in fecundity with mating delay was not found: Hattori and Sato 1983; Kehat and Gordon 1975, 1977. Torres-Vila et al. (2002) implicated an artifact in Kehat and Gordon's rearing methods as a possible explanation for the discrepancy, given that subsequent experiments with each of their same two study species found contradictory results (Ellis and Steele 1982; Kostandy et al. 1992; see Table 3.1). In the one butterfly that has been studied, no reduction in

female reproductive output with mating delay was found (Hiroki and Obara 1997). Despite the near ubiquity of the general pattern of decreased fecundity and fertility with increased female age at mating in moths, the strength of effects varies among species. Thus, implications regarding the underlying biological mechanisms may also vary.

At least six factors may contribute to reduced fecundity or fertility with a delay in mating: (1) decrease in nutritional reserves available for egg development or oviposition, (2) resorption of nutrients from eggs before they are laid (or use of nutrients for other processes instead of egg production), (3) a diminished ability to store or transport viable sperm (Proshold 1996), (4) deterioration in viability of ova with time, (5) interference of oocyte degradation products with sperm migration and/or egg fertilization (Torres-Vila et al. 2002), or (6) laying of unfertilized eggs before mating. One might expect the first two of these non-mutually exclusive possibilities to be particularly important for a species like oriental beetle in which adult feeding is limited. Although I did not directly test explanations (1) or (4), I show here that my data do not support explanations (2) or (6), nor do my data support explanations (3) or (5), which deal specifically with reduced egg fertility from mating delay.

Egg production over the life of the female

The total number of eggs produced by females (number of eggs laid + number remaining at death) did not differ among unmated females and those mated at different ages, suggesting that a cue related to copulation—which may induce egg production in some moths (Proshold et al. 1982)—may be of less importance for total lifetime egg production in oriental beetle. Further, the fact that the total number of eggs recovered

from females (either laid or unlaid) did not differ significantly across age treatments suggests that mating delay did not cause females to resorb nutrients from eggs (see discussion on lifespan below).

In oriental beetle, it is not known how or when egg production is triggered over the female's adult life, but females appear not to possess their full complement of eggs upon reaching sexual maturity. Unmated females had on average ca. 19 eggs remaining in their abdomen at death; however, I have dissected several 5-day-old virgin females and never found more than 5-6 ova per female (data not shown), which suggests that females continue to produce eggs after reaching sexual maturity regardless of whether they mate. It is possible, though, that some cue related to mating causes the rate of egg production to increase. Interestingly, Facundo (1997) found that females collected from flowers in the field (i.e. females that were assumed to have mated and already laid most or all of their eggs) laid a few more eggs when allowed to oviposit in the lab, but when such females were dissected immediately, few or no eggs were found. Thus, additional eggs laid by females might be produced using nutrients derived from flower consumption. The individuals used in my study were not fed, but it would be interesting to see how flower feeding might interact with the effects of delayed mating on reproductive output.

Temporal oviposition patterns

The increase in female egg output per day functioned to partially compensate for mating delay; the compensation was incomplete, however, because later-mating females still laid fewer total eggs than earlier-mated ones. Although larger females produced

and laid more total eggs, they were apparently unable to compensate for mating delay by laying more eggs per day than smaller females.

In moths, females may generally lay nearly all eggs that have matured during the period between emergence and mating and then continue to lay a smaller quantity of eggs as they are produced (Proshold et al. 1982). My results for oriental beetle were generally consistent with this pattern; females typically laid many more eggs over the day following mating than on any other day (Figure 3.7), and the number of eggs laid on the first day of oviposition was positively correlated with female age at mating ($F = 17.4$, $t = 4.17$, $P < 0.0001$, $r^2 = 0.266$). Females mated at younger ages subsequently laid roughly 1-3 eggs per day after the first day, with oviposition rate gradually declining toward zero, whereas daily oviposition rate for females mated at 11-13 d dropped more sharply toward zero after the first day (Figure 3.7).

Females of many moth species may exhibit reduced reproductive output with mating delay in part as a result of laying a relatively large number of infertile eggs before mating has occurred (e.g., Proshold et al. 1982; Leather et al. 1985; Lingren et al. 1988; Wakamura 1990; Unnithan and Paye 1991; Fadamiro and Baker 1999). Oviposition in unmated oriental beetle, however, rarely occurred, and no individuals that were subjected to mating delay laid any eggs in their rearing cups before mating.

Patterson et al. (1932) found that egg fertility in *Drosophila melanogaster* was lower over the day following mating compared to subsequent days. The authors suggested that eggs may be laid too rapidly for the inseminating mechanism to function properly or that the older of the retained eggs may degenerate. In oriental beetle I found no such change in egg fertility over time when comparing egg fertility on the first day

of oviposition versus fertility over all subsequent days (paired t-test: $t = -0.67$, $P = 0.252$, $n = 44$). Females were apparently able to fertilize eggs at the same rate regardless of the age at which they mated or the number of days following mating. Instead, I show below that for oriental beetle decreased fertile egg output with increased age at mating was strongly dependent upon a limited lifespan and subsequent shorter oviposition period when females were mated at older ages.

Lifespan and duration of pre-oviposition and oviposition period

Delayed mating has been shown to increase adult longevity in many moth species (see Table 3.1), and unmated females especially tend to live longer than females that mate, regardless of the length of delay in mating (e.g., Kehat and Gordon 1977; Ellis and Steele 1982; Proshold et al. 1982; Kiritani and Kanoh 1984; Henneberry and Clayton 1985; Lingren et al. 1988; Unnithan and Paye 1990, 1991; Vickers 1997; Fraser and Trimble 2001). Proshold et al. (1982) suggested that a positive correlation between female longevity and age at mating may reflect a change in the allocation of nutritional reserves after mating. That is, mating initiates (or further stimulates) egg maturation, which depletes nutritional reserves and leaves fewer nutrients to sustain other physiological processes in the female. Similarly, delayed mating might induce a female to resorb nutrients from existing ova, which could function to further longevity at the expense of future fecundity.

Unlike in many Lepidoptera, adult lifespan of oriental beetle did not differ among unmated females and those mated at different ages. Females were apparently not resorbing nutrients from unlaid ova (see above) and translating these nutrients into a

longer lifespan. Females appear to produce eggs over their adult life regardless of mating status and do not respond to delayed mating by reallocating resources such that lifespan is altered. Interestingly, I did find a positive, albeit weak relationship between female size and lifespan, suggesting that general nutritional reserves (rather than reabsorbed nutrients from eggs) might contribute to female longevity.

Leather (1988) proposed that in the Lepidoptera, adult female longevity (and the factors influencing it) may be the single most important factor impacting fecundity—even more so than female size, the influence of which may be more obscured by other factors such as environment, diet, and genetics. Fecundity did increase linearly with lifespan duration (full model with mating age included: $F = 18.4$, $P < 0.0001$, $r^2 = 0.401$; partial regression slope: $t = 3.9$, $P = 0.0003$), but again, female longevity was not related to age at mating. It could be that increased lifespan from mating delay has not evolved in oriental beetle because of the ecology of the species, wherein populations are patchy, but dense, and dispersal is limited. The typically close proximity among individuals in natural populations as well as male-biased operational sex ratios might make substantial delays in mating unlikely in nature. It should be noted, however, that the ecology of oriental beetle in non-adventive situations is unknown, and might not be characterized by such high local population densities.

Although early work with oriental beetle in Hawaii (Bianchi 1935) reported the pre-oviposition period to be rather variable (ranging from 1-11 d, $n = 12$), my results showed that the pre-oviposition period—at least for the populations I studied—is generally quite short, and rarely more than 3 days. In fact, my data represent an overestimate of the pre-oviposition period, because I checked oviposition cups for the

first time ca. 36 h after mating, thereby making 1.5 d the lowest possible recorded value for the pre-oviposition period. Only six individuals took more than 1.5 d to begin laying eggs and only two individuals took more than 2.5 d. Other than bias from his small sample size or perhaps genetic drift, an explanation for this discrepancy between my results and those of Bianchi (1935) escapes me. However, a female that could begin laying eggs as soon as possible after mating would likely realize benefits in terms of greater than average lifetime fecundity, especially given the relatively short adult lifespan of oriental beetle and the strong links between oviposition period and fecundity. Although the pre-oviposition period did not differ among age treatments, it is worth noting that every fertile female mated at age 11-13 d had begun ovipositing by the first time I checked for eggs (1.5 d), but there was some variance in duration of the pre-oviposition period for most of the younger age treatments. A trend toward a shorter pre-oviposition period with increased delay in mating is consistent with the general tendency of females mated at older ages (among those that were fertile) to increase egg output. My results for earlier-mated females are similar to the results reported by Bianchi (1935) for several other parameters relating to female reproductive performance, including: fecundity, eggs laid per day, total egg production, lifespan, and mean duration of oviposition period.

In general, females—especially those mated at older ages—continued to lay eggs up until death. Thus, because neither the pre-oviposition period nor adult lifespan differed with delay in mating, the oviposition period declined with increased mating delay simply because oviposition began later for females mated at older ages and ended at death. Females apparently produced the same number of eggs regardless of mating

delay (see above), but females mated at older ages had less time available to oviposit and, therefore, had more eggs remaining at death. For moth species in which the effect of delayed mating on duration of the oviposition period has been examined, oviposition duration nearly always declines with mating delay (Walker 1991; Unnithan and Paye 1990, 1991; Rojas and Cibrian-Tovar 1994; Barrer 1976; Spurgeon et al. 1997; Fraser and Trimble 2001; Torres-Vila et al. 2002). As in oriental beetle, this pattern apparently is simply a reflection of a finite lifespan and reduced available time for laying eggs when mated at an older age. However, unlike in oriental beetle, decreased oviposition period with mating delay in moths is often partially offset by increased lifespan.

Management implications

In contrast to many species of moths, oriental beetle was relatively resilient to the potentially negative impacts of delayed mating. That is, although fecundity was negatively correlated with mating delay, only in females older than nine days was fecundity notably reduced. Moreover, egg fertility was unrelated to female age at mating, and only in females older than 10 d did female fertility decline. Thus, from a management perspective, a six or more day delay in mating would be required in order for the benefits of decreased fecundity and female fertility to be realized. A six day mating delay might be unlikely in a dense infestation of oriental beetle (but see Chapter 2), particularly considering the species' aggregative distribution. Despite the bleak picture painted by this paragraph, several points regarding oriental beetle biology should be considered further.

First, females release peak levels of pheromone at 5 d and progressively less each day thereafter (up to at least 8 d; Zhang et al. 1994); therefore, older females are almost certainly less attractive to males. My experimental design ensured that even older females mated because of the simplicity of the laboratory environment and the close proximity of the introduced male. All females called, but older ones did so with less vigor (personal observation), and males tended to take longer to begin mounting older females, sometimes walking over the female several times with antennae splayed before mounting (personal observation); thus, older females may have produced less contact pheromone. In the complexity of the field environment, older females might be overlooked by potential mates. Therefore, it is probable that the longer a female goes without mating, the less likely she will attract a mate.

Second, Facundo (1997) found that, depending on soil depth and composition, females may take 1-5 d to reach the soil surface after reaching sexual maturity in their pupal cells. Even though females are sexually mature at 4-5 d, duration of mating delay in the field may be an additive combination of the delay in reaching the soil surface to call as well as any possible delay from mating disruption. Thus, delaying mating until a female is $\geq$ 11 d may be likely.

Third, females laid more eggs per day with increased delay in mating; therefore, a delay in mating might result in females laying a high density of eggs in an even more localized area than they otherwise would. Such a situation could contribute to localization of infestations and make mating disruption work well with other management practices (e.g., biological control agents that rely on contact or close proximity between individuals for transmission).

Fourth, although females generally call, mate, and deposit eggs within a very small radius from their point of emergence from the soil (Facundo et al. 1999a), it is possible that they will disperse if they have not been mated for some time. Whether any possible increase in female dispersal is beneficial in terms of management will depend on how far females disperse and whether they are likely to move off the bog or to another part of the bog. In any event, unmated females will be on the soil surface for a longer time and will likely be subjected to greater risk of predation.

Finally, I suggested above that the clumpy distribution of oriental beetle might undermine mating disruption by facilitating more chance encounters between the sexes. However, because males may be responsive to pheromone 24 hours per day (Facundo et al. 1999b), if upon emergence males disperse in response to the presence of synthetic sex pheromone, many males may be unlikely to be in the same vicinity from which they emerged when females in that area are calling.

Though the intensity and nature of the effects may differ among species, decreased reproductive output with delay in mating is widespread across Lepidoptera, and possibly for other insect groups. Laboratory studies will continue to be an important first step toward assessing the potential importance of delayed mating in mating disruption. However, many factors may influence mate finding and reproduction in the field (see above; Sanders 1997), and moth species that are very susceptible to the negative impacts of delayed mating in laboratory studies have not necessarily been managed successfully by mating disruption and vice versa (Cardé and Minks 1995). Thus, for oriental beetle—as well as for the many moth species in which

the effects of female age at mating have been studied in the lab—it will be useful to investigate the importance of delayed mating in the field (see Chapter 2).

Conclusions: delayed mating beyond the Lepidoptera

Delayed mating studies are limited almost exclusively to the Lepidoptera; I am aware of only a few other arthropod groups in which the impacts of delayed mating on female reproductive output were directly studied (see Table 3.1). Among a few scale insects (Bonaponte 1981; Bourijate and Bonaponte 1982) and ticks (Sonenshine 1967; Randolph 1980; Chilton et al. 1992; Chilton et al. 1993), the effects age at mating on reproductive output of females were quite variable among species, with some species exhibiting no negative effects with mating delay, at least for the ages studied. Delayed mating has also been studied in *Drosophila melanogaster*, which showed a moderate decline in fecundity but no change in fertility with mating delay (Redfield 1966). The limited interest in delayed mating for arthropod groups other than Lepidoptera is not entirely surprising, considering the primary aim in studying the effects of mating delay on female reproductive output is based on the implications for mating disruption. However, it is interesting to note that of the few non-lepidopterans studied so far, the effects on reproductive output of females appear not to be as pronounced as in moths. Interest in the use of semiochemicals for disruption of insect orientation behavior exists beyond the Lepidoptera. For example, mating disruption has been studied in scolytine bark beetles (Coleoptera: Curculionidae; Trudel et al. 2004), plant bugs (Heteroptera: Miridae; Kakizaki 2004), and sawflies (Hymenoptera: Diprionidae; Östrand et al. 1999). As the exploration of mating disruption in the management of non-lepidopteran

insects will undoubtedly increase, interest in the effects of delayed mating in these groups will also expand. It will be interesting to see whether the general patterns found in moths with respect to the effects of mating delay on female reproduction will be applicable across other insect orders. My study represents the first report of the effects of delayed mating on the reproductive output of a coleopteran species.

Table 3.1. Summary of literature on the effects of delayed mating on female insects.

Order	Family	Species	fecundity	egg fertility	female fertility	pre-oviposition period	oviposition period	lifespan
Diptera	Drosophilidae	*Drosophila melanogaster*[1]	-					
Homoptera	Diaspididae	*Aonidiella aurantii*[2]	=				-	
		Chrysomphalus ficus[3]	-					
		Lepidosaphes beckii[2]	=				-	
		Parlatoria pergandei[2]	-				-	
		Parlatoria zizyphus[2]	-				-	
Lepidoptera	Cranbidae	*Ostrinia nubilalis*[4]	-	-	-	+		+
	Gelechiidae	*Pectinophora gossypiella*[5]	-	=	-			+
		Pectinophora scutigera[6]	-	=	-	=	-	=
	Lymantriidae	*Lymantria dispar*[7]	-	-				=
	Lyonetiidae	*Leucoptera coffeella*[8]	-	-				
	Noctuidae	*Busseola fusca*[9]	-	-		+	-	+
		Chilo partellus[10]	-	-	-	+	-	+
		Copitarsia consueta[11]	-	-		+	-	
		Earias insulana[12]	=	=	=	+		+
		Earias insulana[13]	-					
		Heliothis virescens[14]	-	=	-			+
		Panolis flammea[15]	-	-		+		=
		Panolis flammea[16]	-	-				=
		Spodoptera exigua[17,18,19]	-	-	-			+
		Spodoptera frugiperda[20,21]	-	-				+
		Spodoptera littoralis[22]	=	=			+	+
		Spodoptera littoralis[23]	-	-	-	+		+
	Phycitidae	*Ephestia cautella*[24]	-	-			-	+
	Pieridae	*Eurema hecabe*[25]	=	=		-	=	=
	Pyralidae	*Cadra cautella*[26]	-	-				
		Cryptoblabes gnidiella[27]	-	-				
		Eoreuma loftini[28]	-	-		-	-	=
		Ephestia kuehniella[29]	-	-				+
		Etiella zinckenella[30]	=					+
		Plodia interpunctella[31]	-	-	-	+		
	Tortricidae	*Adoxophyes orana*[32]	-	-				+
		Choristoneura rosaceana[33]			-			
		Cnephasia jactatana[34]	-	-				
		Cryptophelbia illepida[35]	-	-		=		=
		Cydia pomonella[29,36]	-	-	=		-	+
		Cydia pomonella[37]	-	-				+
		Epiphyas postvittana[38]	-		-			
		Grapholita molesta[39]	-	-	-	-	-	+
		Homona magnanima[40]	-	-				+
		Lobesia botrana[41]	-	-	-		-	+
	Yponomeutidae	*Yponomeuta cognagellus*[29]	-	=				+

Table 3.1. (continued)

References: 1. Redfield 1966; 2. Bourijate and Bonaponte 1982; 3. Bonafonte 1981; 4. Fadamiro and Baker 1999; 5. Lingren et al. 1988; 6. Walker 1991; 7. Proshold 1996; 8. Michereff et al. 2004; 9. Unnithan and Paye 1990; 10. Unnithan and Paye 1991; 11. Rojas and Cibrian-Tovar 1994; 12. Kehat and Gordon 1977; 13. Kostandy et al. 1992; 14. Proshold et al. 1982; 15. Leather et al. 1985; 16. Leather and Burnand 1987; 17. Wakamura 1990; 18. Rogers and Marti 1997; 19. Rogers and Marti 1996; 20. Rogers and Marti 1994a; 21. Rogers and Marti 1994b; 22 Kehat and Gordon 1975; 23. Ellis and Steele 1982; 24. Barrer 1976; 25. Hiroki and Obara 1997; 26. Amoako-Atta and Mills 1977; 27. Wysoki et al. 1993; 28. Spurgeon et al. 1997; 29. Karalius and Būda 1995; 30. Hattori and Sato 1983; 31. Huang and Subramanyam 2003; 32. van der Kraan and van der Straten 1988; 33. Delisle 1995; 34. Jiménez-Pérez and Wang 2003; 35. Jones and Aihara-Sasaki 2001; 36. Vickers 1997; 37. Knight 1997; 38. Foster and Ayers 1996; 39. Fraser and Trimble 2001; 40. Kiritani and Kanoh 1984; 41. Torres-Vila et al. 2002.

Note: Plus sign (+) denotes variable increased, minus sign (-) denotes variable decreased, and equals sign (=) denotes variable was not affected by delayed mating. Symbols are absent where data were unavailable or unclear. Decreased female fertility in at least some cases resulted from failed mating (i.e. sperm was not transferred). Differences in lifespan reflect differences between virgin and mated females, between females mated at different ages, or both. Foster and Ayers (1996) reported a decline in the number of fertile eggs laid by *Epiphyas postvittana* with increased age at mating, but did not specify whether this decline reflected a decrease in fecundity, fertility, or both.

Table 3.2. ANOVA of each dependent variable as a function of female age at mating and female size (estimated by right elytron length).

A. Dependent variable: total number of eggs laid

Source of variation	F	P	r^2	Estimate	SE	t	P
Model	63.4	<0.0001	0.779				
age, linear				-1.47	0.18	-7.95	<0.0001
size, linear				0.33	0.07	4.67	<0.0001
age, quadratic				-0.35	0.07	-5.17	<0.0001

B. Dependent variable: total egg production (number of eggs laid + number of eggs remaining at death)

Source of variation	F	P	r^2	Estimate	SE	t	P
Model	12.3	<0.0001	0.308				
age, linear				0.16	0.27	0.59	0.555
size, linear				1.26	0.26	4.92	<0.0001

C. Dependent variable: number of eggs remaining in abdomen at death

Source of variation	F	P	r^2	Estimate	SE	t	P
Model	725.8	<0.0001	0.964				
age, linear				1.88	0.05	36.3	<0.0001
age, quadratic				0.24	0.02	11.4	<0.0001

D. Dependent variable: number of eggs laid per day

Source of variation	F	P	r^2	Estimate	SE	t	P
Model	2267.3	<0.0001	0.979				
age, linear				0.91	0.02	47.6	<0.0001

E. Dependent variable: adult lifespan

Source of variation	F	P	r^2	Estimate	SE	t	P
Model	2.3	0.111	0.077				
age, linear				-0.08	0.11	-0.75	0.459
size, linear				0.22	0.11	2.00	0.051

Table 3.2. (continued)

F. Dependent variable: oviposition period

Source of variation	F	P	r^2	Estimate	SE	t	P
Model	312.6	<0.0001	0.867				
age, linear				-0.69	0.04	-17.7	<0.0001

The partial regression slopes are shown only for the parameters that comprised the final model. I manually removed each term from the initial model that was not statistically significant ($\alpha = 0.05$) or nearly so ($\alpha = 0.10$); however, female age at mating was retained in every model, even if its effects were not statistically significant.

Table 3.3. Mean ± SE egg fertility and duration of pre-oviposition period, as well as mean female fertility across age treatments. Differences among means for egg fertility and pre-oviposition period were compared by Kruskal-Wallis tests.

Female age at mating	N	Egg fertility (%)	Pre-oviposition period (d)*	Female fertility (%)
Unmated	7	—	—	—
Clutch treatment	5	(90.5 ± 5.3)	—	(100)
4 d	6	92.1 ± 5.3	1.5 ± 0.0	100
5 d	7	97.1 ± 1.0	2.5 ± 1.0	86
6 d	7	94.2 ± 4.5	1.6 ± 0.14	100
7 d	6	98.7 ± 0.8	1.5 ± 0.0	100
8 d	6	92.0 ± 6.4	1.7 ± 0.17	100
9 d	6	73.0 ± 17.0	1.7 ± 0.17	100
10 d	5	92.2 ± 6.5	2.3 ± 0.58	100
11 d	5	87.7 ± 0.64	1.5 ± 0.0	60
12 d	5	95.0 ± 2.8	1.5 ± 0.0	60
13 d	5	76.1 ± 24	1.5 ± 0.0	40
	Test statistic	$\chi^2 = 8.49$	$\chi^2 = 6.81$	Fisher's exact test
	P-value	0.496	0.657	0.008

Means in parentheses are shown for comparison, but the data for these treatments were not included in analyses.

*Egg production was checked for the first time ca. 36 h after mating, so the minimum length of the pre-oviposition period was 1.5 d.

Table 3.4. Mean ± SE total egg production, lifespan, and number of eggs remaining in the abdomen at death across age treatments. Differences among means were compared by ANCOVA with female elytron length as the covariate.

Female age at mating	n	Total egg production	Lifespan (d)	Eggs at death
Unmated	7	20.3 ± 3.4	15.7 ± 1.3	19.4 ± 3.0
Clutch treatment	5	(28.4 ± 2.6)	—	(0.0 ± 0.0)
4 d	6	23.2 ± 2.8	15.8 ± 1.4	0.67 ± 0.33*
5 d	7	25.7 ± 2.4	17.0 ± 0.53	4.3 ± 3.1*
6 d	7	23.9 ± 2.4	15.6 ± 1.1	2.6 ± 1.8*
7 d	6	27.3 ± 3.2	16.3 ± 1.4	3.5 ± 1.4*
8 d	6	23.0 ± 1.6	15.0 ± 1.1	2.5 ± 2.5*
9 d	6	25.8 ± 4.4	15.2 ± 1.0	6.2 ± 3.3*
10 d	5	24.6 ± 2.0	14.8 ± 0.86	9.0 ± 2.9*
11 d	5	18.2 ± 1.4	15.0 ± 0.89	8.4 ± 5.2*
12 d	5	29.2 ± 3.3	14.8 ± 0.80	18.2 ± 4.7
13 d	5	27.0 ± 2.8	17.0 ± 0.89	19.4 ± 5.4
	Test statistic	F = 1.20	F = 0.99	F = 205.1
	P-value	0.311	0.460	<0.0001

Means within a column followed by an asterisk differ significantly from the unmated female treatment (Dunnett's test, $\alpha = 0.05$). Means in parentheses are shown for comparison, but the data for these treatments were not included in analyses.

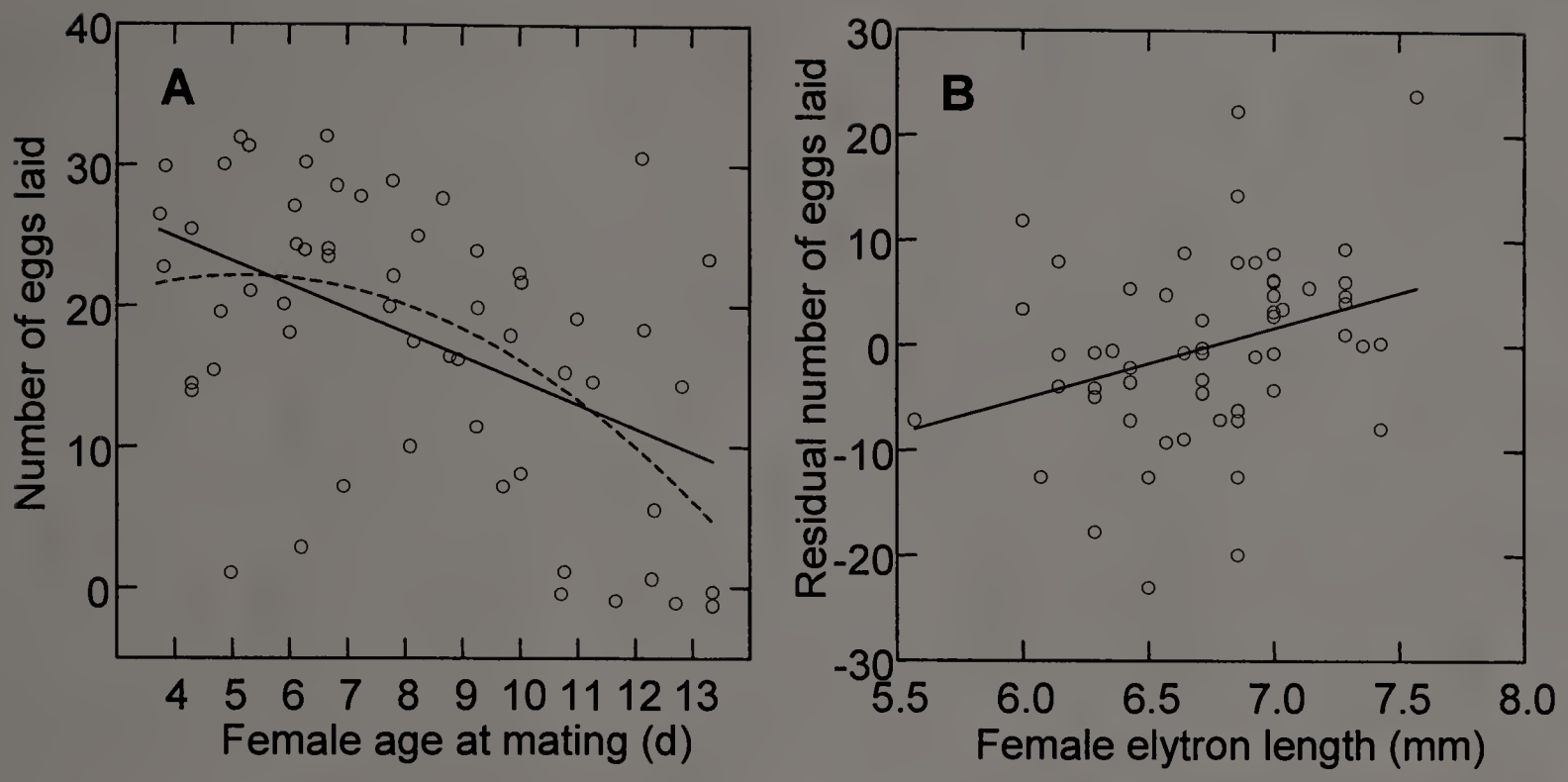


Figure 3.1. Effect of female age at mating on fecundity (A) and effect of female size (as estimated by right elytron length) on fecundity, accounting for effects of female age at mating (B). Plotted in (B) is the relationship between female size and the deviation from expected fecundity due to female age at mating (i.e. the residuals following the regression of fecundity on mating age). Points in (A) were randomly jittered slightly to reduce overlap.

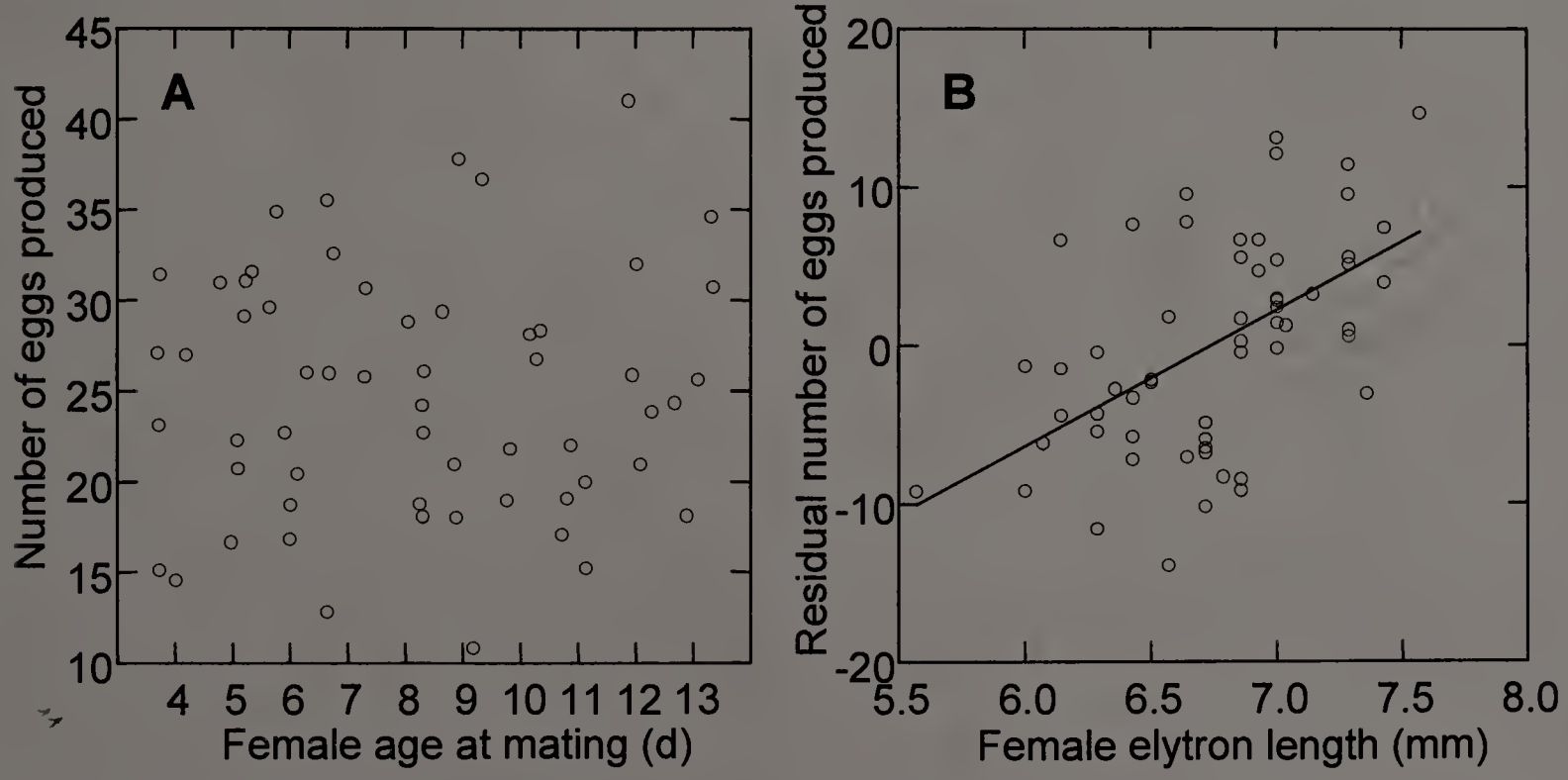


Figure 3.2. Effect of female age at mating on total egg production (A) and effect of female size (as estimated by right elytron length) on total egg production, accounting for effects of female age at mating (B). Plotted in (B) is the relationship between female size and the deviation from expected egg production due to female age at mating (i.e. the residuals following the regression of egg production on mating age). Points in (A) were randomly jittered slightly to reduce overlap.

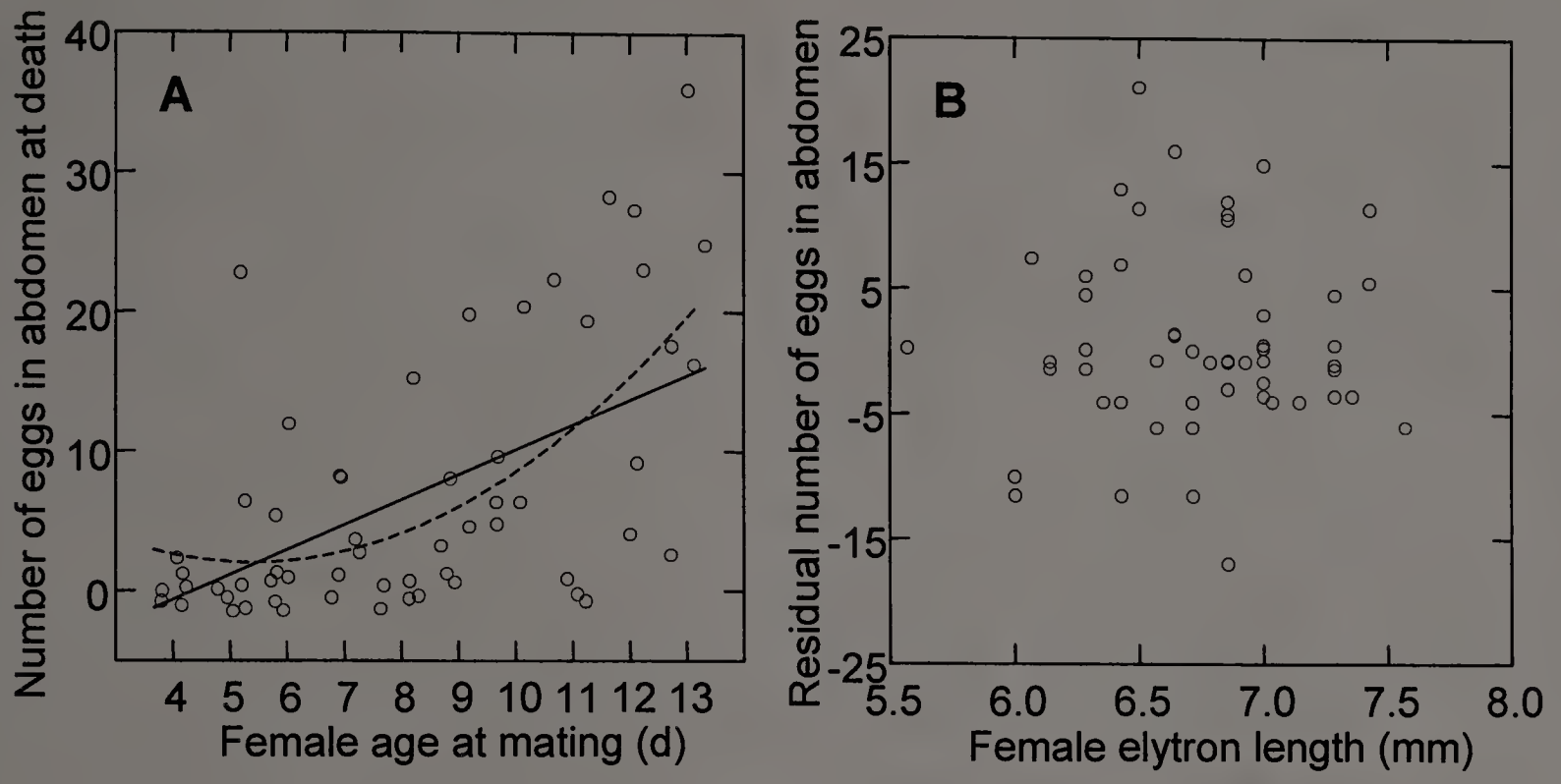


Figure 3.3. Effect of female age at mating on the number of eggs remaining in the abdomen at death (A) and effect of female size (as estimated by right elytron length) on the number of eggs remaining in the abdomen, accounting for effects of female age at mating (B). Plotted in (B) is the relationship between female size and the deviation from expected eggs in abdomen due to female age at mating (i.e. the residuals following the regression of eggs in abdomen on mating age). Points in (A) were randomly jittered slightly to reduce overlap.

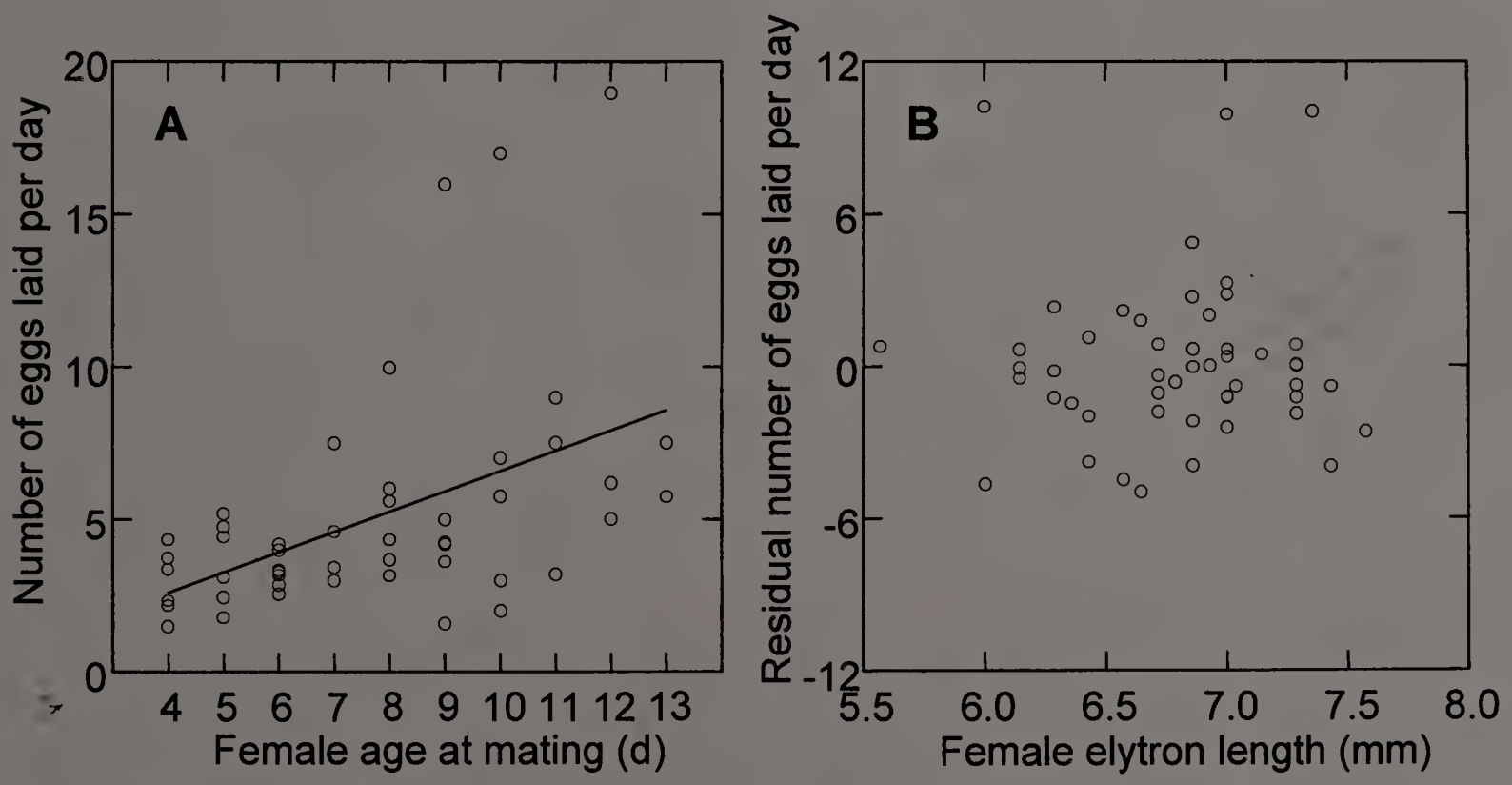


Figure 3.4. Effect of female age at mating on the number of eggs laid per day (A) and effect of female size (as estimated by right elytron length) on the number of eggs laid per day, accounting for effects of female age at mating (B). Plotted in (B) is the relationship between female size and the deviation from expected eggs laid per day due to female age at mating (i.e. the residuals following the regression of eggs laid per day on mating age).

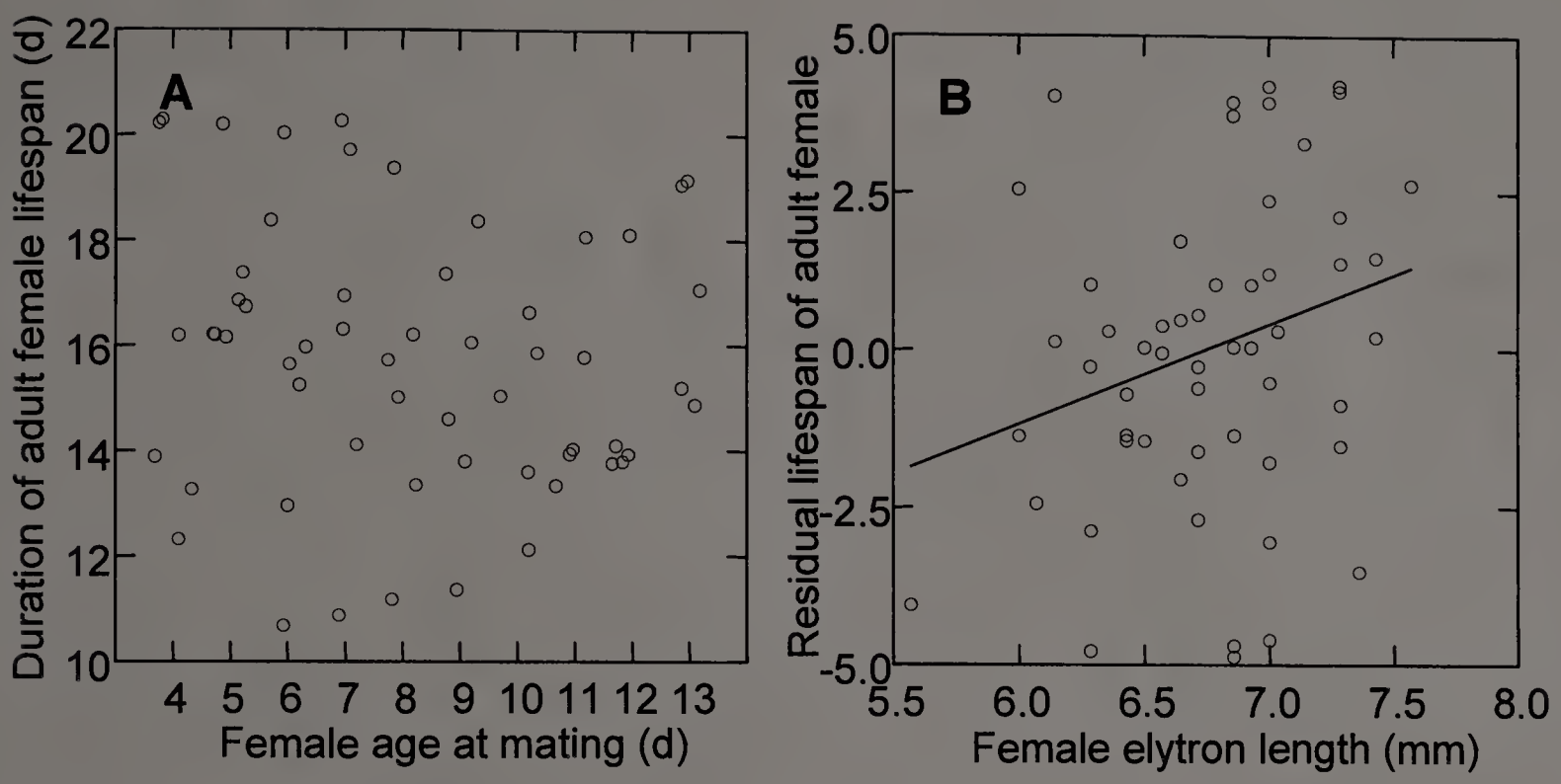


Figure 3.5. Effect of female age at mating on longevity (A) and effect of female size (as estimated by right elytron length) on longevity, accounting for effects of female age at mating (B). Plotted in (B) is the relationship between female size and the deviation from expected longevity due to female age at mating (i.e. the residuals following the regression of longevity on mating age). Points in (A) were randomly jittered slightly to reduce overlap.

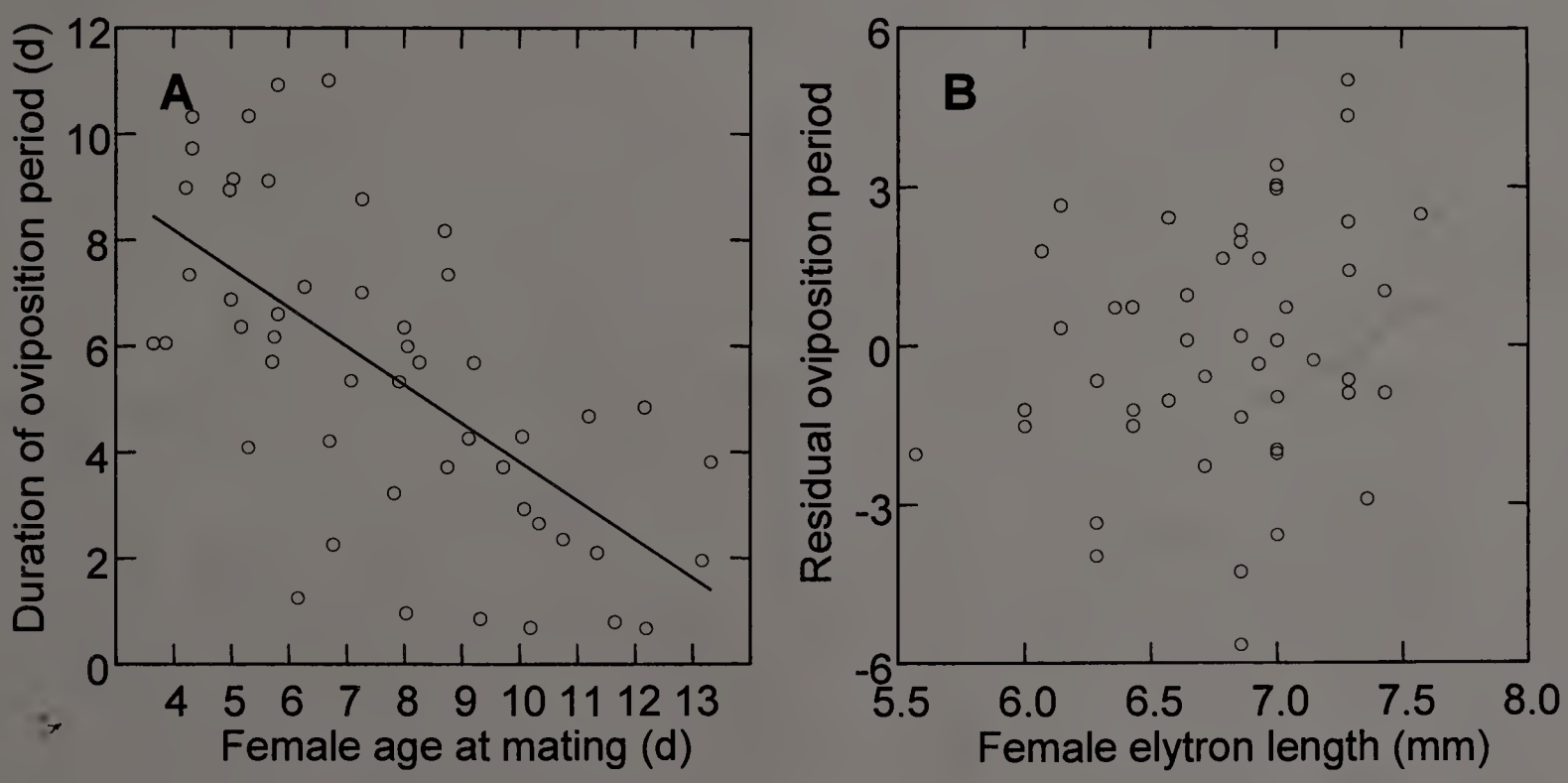


Figure 3.6. Effect of female age at mating on duration of the oviposition period (A) and effect of female size (as estimated by right elytron length) on oviposition period, accounting for effects of female age at mating (B). Plotted in (B) is the relationship between female size and the deviation from expected oviposition period due to female age at mating (i.e. the residuals following the regression of oviposition period on mating age). Points in (A) were randomly jittered slightly to reduce overlap.

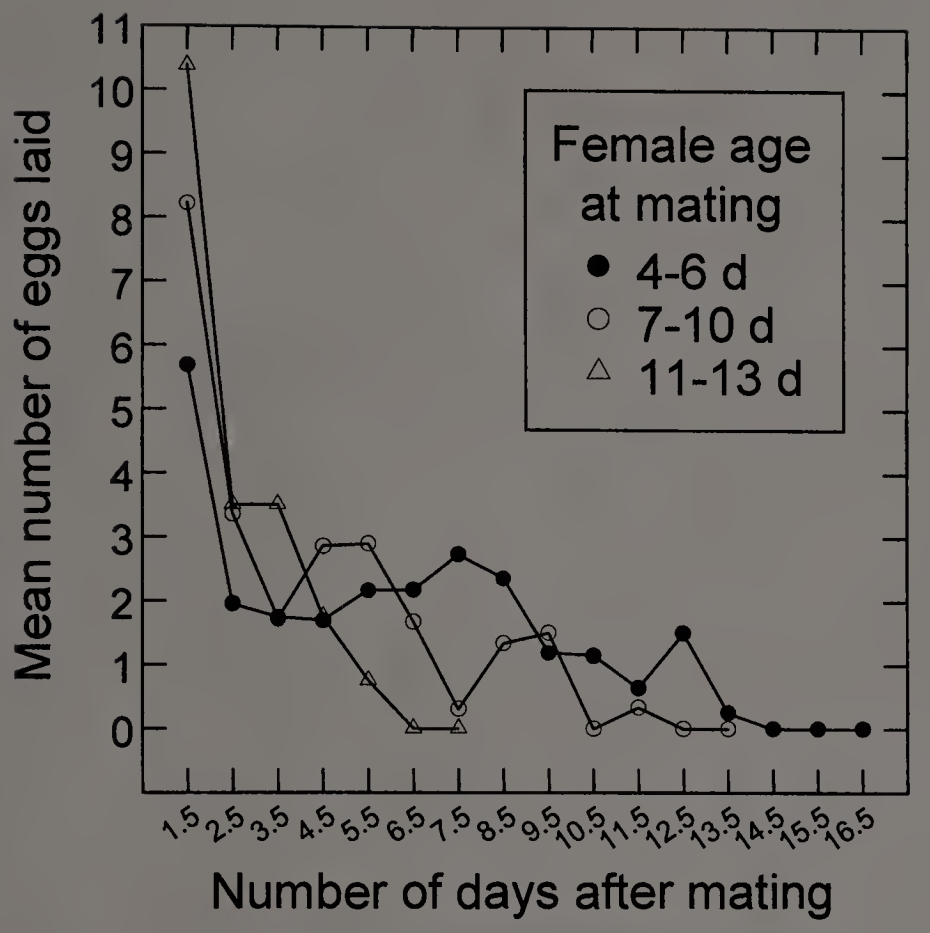


Figure 3.7. Temporal patterns of oviposition. Treatments were subjectively grouped based on similar numbers of eggs laid per day. The number of eggs laid by each female was counted 36 h after mating and every 24 h thereafter.

CHAPTER 4

THE INFLUENCE OF BODY AND GENITALIA MORPHOLOGY ON RELATIVE MALE FERTILIZATION SUCCESS IN ORIENTAL BEETLE: SIZE MATTERS, BUT IS BIGGER BETTER?

Abstract

Although the frequently large variance in relative male fertilization success when females are mated by more than one male has been appreciated for some time, the factors that influence relative paternity are still poorly understood. Recently, experimental evidence that morphology of male genitalia influences fertilization success has been documented in water striders, a dung beetle, and a leaf beetle. I explored the role of male genital morphology in post-copulatory sexual selection in oriental beetle. I mated females to two males in succession and assessed relative paternity by the sterile male technique. Morphology of the male genitalia was found to strongly influence relative paternity, but only for the first male to mate. Male body size influenced relative fertilization success as well, but again, only for the first male; surprisingly, smaller males achieved higher paternity when mating first. I also found suggestive evidence that copula duration of both the first and second male to mate influenced paternity. Other factors, including female size and degree of asymmetry of hind tibiae length of males had no effect on relative fertilization success. My results for oriental beetle are novel among sperm precedence studies for two reasons: (1) traits of the first male appear to be more important in influencing paternity than those of the second, and (2) smaller, not larger, males achieved greater relative paternity. My

results also contribute to the growing body of empirical evidence in support of the hypothesis that male genitalia evolve by post-copulatory sexual selection.

Introduction

Following Parker's (1970) seminal review, the study of sperm precedence (i.e. the relative fertilization success among males in successive matings of the same female) has received considerable attention. That male mating success does not necessarily correlate directly with reproductive fitness when females mate multiply is apparent from the large variance in sperm precedence found in many insect species (Simmons 2001). The significance of this variance has long been appreciated (e.g., Lewis and Austad 1990; Simmons and Parker 1992); however, the factors that influence relative paternity are still poorly understood. The determination of what kinds of male traits are favored by post-mating sexual selection is still a crucial issue in sexual selection theory.

Of the traits that influence sperm precedence in insects, the two most widely documented are copula duration and male body size (reviewed by Simmons 2001). Longer copulation might facilitate: (1) transfer of more sperm (e.g., Dickinson 1986; Simmons and Parker 1992; Arnqvist and Danielsson 1999b) or (2) accessory gland products (Parker 1970; Wing 1985), (3) removal of more sperm of prior males (e.g., Siva-Jothy 1987), or (4) more stimulation to the female to induce any number of responses, including inhibition of remating or mobilization of sperm (that of the current or of prior males; Eberhard 1996). Similarly, larger males may be better competitors for fertilization because they may produce larger ejaculates (e.g., Fox et al. 1995b; Bissoondath and Wiklund 1996; Schlüns et al. 2003). Given the general advantage of

larger males with respect to pre-copulatory female choice and intrasexual competition for females (Andersson 1994), it is tempting to assume that post-copulatory sexual selection reinforces the action of pre-copulatory sexual selection (i.e. traits that are favored under pre-mating sexual selection are also favored by post-coupling processes like sperm competition and cryptic female choice). Some species do fit the expected pattern [for example, larger *Dryomyza anilis* flies are selected by both pre- (Otronen 1984) and post-copulatory (Otronen 1994) selection], but more direct tests with two model systems—water striders (Arnqvist and Danielsson 1999b) and *Drosophila melanogaster* (Brown et al. 2004; but see Bangham et al. 2002)—do not support the congruence of pre-mating and post-mating sexual selection. For species in which females lack direct pre-copulatory mate choice, post-copulatory sexual selection might be clearer because of the reduction in potentially counteractive forces of sexual selection in operation before coupling (see Sih et al. 2002).

One male trait that may evolve primarily via post-copulatory sexual selection, and is unlikely to be driven directly by pre-copulatory selection, is the morphology of the genitalia (Eberhard 1985, 1994, 1996). Three major hypotheses to explain the rapid and divergent evolution (Eberhard 1985) of male genitalia have been advanced: (1) the lock-and-key hypothesis, which suggests that genitalia evolve via selection for pre-insemination reproductive isolation (reviewed by Shapiro and Porter 1989), (2) the pleiotropy hypothesis (Mayr 1963), which states that genitalia evolve not by direct selection, but via selection on non-sexual but genetically-correlated traits, and (3) the sexual selection hypothesis. Three major models of sexual selection have been proposed: sperm competition (competition among sperm from different males for the

fertilization of a set of ova; Parker 1970), cryptic female choice (paternity biasing resulting from female morphology, physiology, or behavior that occurs after coupling; Eberhard 1985, 1996; Pitnick and Brown 2000), and conflict between the sexes over control of reproduction (Lloyd 1979; Alexander et al. 1997; Arnqvist and Rowe 2002). While the lock-and-key and pleiotropy hypotheses may continue to possess some utility (see Discussion), evidence against these two hypotheses (Eberhard 2004a; Eberhard and Ramirez 2004) and in support of the sexual selection hypothesis (reviewed by Hosken and Stockley 2004) is mounting. In particular, evolution of genital morphology across most arthropods may be better explained by the former two models of sexual selection (sperm competition and cryptic female choice) rather than the model of sexual conflict (Eberhard 2004b); however, it should be noted the three models are not necessarily mutually exclusive in all cases.

The complexity and diversity of insect genitalia are unlikely to have arisen merely for the relatively simple process of sperm transfer (Hosken and Stockley 2004). Indeed, genitalia morphology might be shaped by selection for other functions, which may include copulatory courtship (e.g., Eberhard 1992, 1993a,b; Otronen 1998) and removal of previous males' sperm either directly (e.g., Waage 1979; Yokoi 1990; Haubruge et al. 1999) or indirectly (e.g., Córdoba-Aguilar 1999). The possible influence of male genitalia on sperm precedence may be independent of male size, which is often a poor predictor of genitalia size (Eberhard 1996; Eberhard et al. 1998; Schmitz et al. 2000). Further, while larger body size might be shaped by both natural selection and sexual selection (both pre- and post-copulatory), evolution of male genitalia morphology is more compatible with the model of post-copulatory sexual

selection; therefore, the influence of male genitalia morphology on male reproductive success, including relative paternity, might be particularly important. The significance of the effects of genital morphology on sperm precedence has been appreciated only recently (Arnqvist and Danielsson 1999a; Danielsson and Askenmo 1999; House and Simmons 2003; Rodriquez et al. 2004; see also Otronen 1998), and, therefore, its effects might be more pervasive than current data suggest.

In sperm precedence studies with two males, most insects exhibit on average either strong second male precedence or roughly equal paternity, with a slight bias toward the second male (Simmons 2001, Table 2.3). Characters of the second male to mate are usually most influential on relative paternity; although relative characters between the first and second male may also be important, traits of the first male alone rarely impact paternity. This pattern might be explained, in part, by the ability of the second male to adjust his mating strategy according to the risk of sperm competition when mating with a non-virgin female (e.g., Wedell and Cook 1999; García-González and Gomendio 2004). While "defensive" adaptations in males to protect against future competitors for a female's ova exist as well (Clark et al. 1995; Arnqvist and Danielsson 1999a; House and Simmons 2003), assessing the risk of possible future competition is likely more difficult than assessing more immediate risks to a male's reproductive success.

Oriental beetle, *Anomala orientalis* (Waterhouse) (Coleoptera: Scarabaeidae), is an important pest of turf, ornamentals, and some food crops (Vittum et al. 1999); Facundo et al. (1999a) characterized the emergence, mating, and post-mating behaviors of oriental beetle, but did not examine sperm precedence in this species. In oriental

beetle, adult virgin females begin calling (i.e. raising the abdomen and releasing sex pheromone) immediately upon emergence from the soil (Facundo et al. 1999a). The first male to reach a female mates with her, with no overt pre-copulatory courtship behavior. In the lab, females burrow into soil immediately after mating and do not surface until most if not all of their eggs have been laid (Bianchi 1935; E.J.W., unpublished data). If a second male attempts to mate with the female before she re-enters the soil, she vigorously waves her hind legs, which functions to inhibit the male's ability to mount her, though most rejection attempts are unsuccessful (Facundo 1997) and the female eventually acquiesces. I predicted that the lack of direct pre-copulatory female choice and overt pre-copulatory courtship behavior could contribute to strong post-copulatory sexual selection.

A key prediction of the evolution by sexual selection model regarding male genitalia is that relative male fertilization success is linked with genital morphology (Eberhard 1985). I tested this hypothesis in oriental beetle by evaluating the influence of male genital morphology on relative paternity in twice-mated females. I also investigated the importance of other potential sources of variation in sperm precedence, including male body size, copula duration, and male body symmetry.

Methods

Collection and rearing of beetles

I collected third instar grubs from turf at Bay Pointe Country Club golf course, Onset, MA on 18 May 2004. All grubs were collected into plastic containers with soil, and then taken back to the lab where they were placed individually into lidded, clear

plastic cups (30 mL) with prepared soil. The soil mixture was made by mixing two parts screened sand with one part screened peat moss (1.18 mm sieve). I sterilized the soil in an autoclave (123˚C, 1 kg/cm^2 for 60 min), allowed it to cool, and then added sterilized, deionized water to moisten the mixture to about 12% water by weight. A heavy pinch of grass seed (Pennington® mix comprised of 63% shining star perennial ryegrass, 15% boreal red fescue, 9% kenblue Kentucky bluegrass, 9% blue bonnet Kentucky bluegrass, 2% inert matter, 1.9% other crop seed, and 0.1% weed seeds) was placed on top of the soil in each cup to provide food for the grubs to continue development. I placed cups on 30-cup capacity clear plastic trays and placed each tray into a clear 9.46-liter plastic, re-sealable bag with a damp sponge to reduce moisture loss from cups. Grubs were held in a rearing chamber on a 16:8 L:D cycle at 10°C to slow development.

About one month before adult beetles were needed, I placed trays in a rearing chamber on a 16:8 L:D cycle at 25°C; adult eclosion began after about 3-4 weeks under these conditions. I checked each cup weekly for pupae, and checked pupae daily for adult eclosion in order to establish adult age. Adults were sexed (based on size of antennal lamellae) and held in their rearing cups until needed for mating experiments.

Sperm precedence after double mating

I mated beetles in the lab under conditions that simulated dusk—the period of peak female sexual receptivity (Facundo et al. 1994) and peak male responsiveness to pheromone (Facundo et al. 1994; E.J.W., unpublished data). I moved beetles to a room held at about 60% humidity and maintained on a 16:8 L:D cycle (as above for rearing

chambers), but at 25-27°C during photophase and 21-22°C during scotophase and the last two hours of photophase. Light intensity during photophase was held at ca. 150 lux, except during the first and last two hours of photophase when it was held at 15 lux. I paired beetles within the last two hours of photophase.

I held virgin females for 5-8 d after eclosion before pairing with 4- to 9-day-old virgin males; females reach sexual maturity by age 5 d, and males age 4-9 d display peak response to female sex pheromone (Zhang et al. 1994). For mating experiments, I placed a female in a plastic, 30mL cup with a layer of soil ca. 0.5 cm deep. Females typically begin calling within 2 min of being placed on soil under simulated dusk conditions (personal observation). I introduced a male after the female began calling; males promptly mounted calling females in the lab (personal observation).

Each female was paired with one sterilized male (S) and one un-sterilized, fertile male (F); females were paired first with either a sterilized male (SF) or a fertile male (FS), with treatments randomly assigned to each female. The second male was introduced immediately after the first pair separated (usually < 1 min after copulation ended). Each sterilized male was made so 3-6 h before use in mating experiments by exposure to gamma radiation (80 Gy at ca. 7.5 Gy/min) using a cobalt-60 source housed in a Gammacell 220 irradiator.

After each mating pair separated, I removed the male and froze him for later morphological measurements (see below); after the female's second mating, I transferred her to a clear, plastic oviposition cup (diameter: 12 cm, height: 13 cm) with a layer of soil ca. 3 cm deep. Ten days after mating (when most if not all eggs had been laid), I sorted through the soil in oviposition cups and collected all eggs into Petri dishes

lined with filter paper moistened with sterilized, deionized water. If the female was still alive, I returned her to the oviposition cup with fresh soil and collected any additional eggs ca. seven days later. I held eggs at least until fertility could be assessed; fertile eggs increase in size, become more spherical, maintain their white color, and just before hatching, the orange-brown mandibles of the developing larva are visible through the chorion (personal observation).

I recorded the percent fertility of each female's clutch of eggs and calculated P_2 (the portion of eggs laid by a female that were sired by the second male to mate) values based on the formula of Boorman and Parker (1976):

$$P(s) = \left(1 - \frac{x}{p}\right) + \left(\frac{z}{p} \times \frac{1 - x/p}{1 - z/p}\right)$$

where P(s) = proportion of ova fertilized by a sterile male, p = fertility after a fertile double mating (FF), z = fertility after a sterile double mating (SS), and x = proportion of eggs that hatch after a double mating FS or SF. Values of P_2 calculated from the Boorman and Parker (1976) equation may be greater than one or less than zero when estimates of x are lower than z or higher than p; P_2 values were transformed using the formula by Cook et al. (1997) so that the data lay within the range of zero to one.

Mating behaviors

For each mating pair, I recorded (in minutes): pre-copulatory "foreplay" duration (length of time between initial contact and intromission), copula duration (length of time between intromission and retraction of the aedeagus), and duration of "resistance" (total length of time the female walked and kicked her hind legs at the copulating male).

Morphological measurements

After use in mating experiments, I measured the dry mass of each male to the nearest 0.1 mg. I measured the length of the left (L) and right (R) hind tibia of each male to the nearest 0.02 mm using a stereo-microscope equipped with an ocular micrometer; I then calculated symmetry of the hind tibiae as the difference between left tibia length and right tibia length (L – R). I also characterized genital morphology of each male using four measurements (see Figure 4.1). To prepare genitalia for measurements, I removed the phallus of each male and macerated it in 10% KOH for 2 h, then in 80% aqueous lactic acid for 10 min before mounting the structures on slides with glycerin. I captured digital images of the genitalia and used ImageJ software (Rasband 1997-2005) to measure the area bound by readily distinguishable landmarks or by traced edges of each sclerite (see Figure 4.1).

Data analysis

To avoid including highly correlated variables within the regression model, I first produced a correlation matrix of all the morphological traits of interest (Table 4.1); although several traits were correlated, the magnitude of any correlations was small and, thus, no traits were excluded. I used multiple linear regression with P_2 value (arcsine transformed to achieve normality) as the dependent variable and the following independent variables (for both the first and second male to mate): male mass, copula duration, $|FA|$ (fluctuating asymmetry) of hind tibiae, and size of the four genital sclerites measured (see Figure 4.1). I also included female size (as estimated by right

elytron length) in the regression model as well as duration of female resistance to the second male (cube root transformed to achieve normality). Finally, I included treatment (FS or SF) in the model using dummy variable coding. I used hierarchical partitioning with Schwarz Bayesian Information Criterion to select the most parsimonious model. All data were analyzed using SAS (version 8.2, SAS Institute, Inc.).

Results

Oriental beetle exhibited mixed paternity after double mating, with sperm precedence ranging from complete first male to complete second male paternity (Table 4.2). The mean P_2 value for FS + SF treatments was 0.58 and did not differ significantly from 0.5 (Wilcoxon signed-rank test, $z = 1.16$, $n = 33$, $P = 0.247$); however, P_2 values were more or less bimodally distributed for SF treatments and normally distributed for FS treatments (Figure 4.2).

The multiple regression analysis revealed two male characters to be strongly correlated with relative paternity: genital spicule size and body mass, both only of the first male to mate (Table 4.3). Relative paternity was also influenced by treatment (FS v. SF; Table 4.3), reflecting a slight negative impact of sterilization on male mating ability—an unfortunate, but common side effect of the sterile male technique (e.g. Danielsson and Askenmo 1999; Vermette and Fairbairn 2002; reviewed by Simmons 2001). P_2 values were negatively correlated with spicule size and positively correlated with the mass of the first male to mate; thus, the larger the spicule and the smaller the first male, the greater his relative paternity (Figure 4.3). Male mass and spicule size were not correlated (see Table 4.1), thus their effects on relative paternity were

independent. Surprisingly, only characters of the first male to mate were correlated with paternity; based on the multiple regression, P_2 values showed no relationship with the second male to mate for any of the characters measured in this study. However, my results provide suggestive evidence that copula duration of both the first and second male to mate may also have had some influence on relative fertilization success. P_2 values tended to decline with increasing duration of copulation of the first male, especially for FS treatments (Figure 4.4); however, the relationship was not significant (full model: $F_{4,32} = 4.61$, $P = 0.006$, $r^2 = 0.397$; partial regression slope: $t = -0.30$, $P = 0.769$), owing largely to two SF data points that were outliers with respect to the overall pattern (see Figure 4.4-A). Although copula duration of the second male appeared not to influence relative paternity, separate analysis of treatments revealed a positive correlation between copula duration and P_2 for the FS treatment (Figure 4.4-B; full model with mass and spicule size of first male: $F_{3,15} = 9.56$, $P = 0.002$, $r^2 = 0.705$; partial regression slope: $t = 2.53$, $P = 0.026$). Asymmetry of the hind tibiae of males was not correlated with relative paternity, nor was duration of female resistance behavior, female size, or mean size of the two spinose patches (the other genital trait included in the analysis).

Copula duration was significantly longer for the second male only in FF treatments (Table 4.4), but second males took longer to achieve intromission ("foreplay" time) for all treatments except for SS (which featured a small sample size for making such comparisons; Table 4.4). Mean duration of female resistance behavior was longer for the second mating across all treatments, although the difference was only marginally significant for the SF treatment (Table 4.4). Male spicule size did not differ

between first and second males (Table 4.4); however, randomization of treatments did not effectively control for differences in male body size. For the SF treatment, first males were significantly larger, and for FS treatment, second males tended to be larger (although the difference was not significant; Table 4.4).

Discussion

The results presented here suggest that at least two male traits contribute to relative fertilization success in oriental beetle: genital morphology and body size of the first male. Here, I show that—unlike in most insect species studied to date—in oriental beetle, traits of the first male to mate appear to have primacy in paternity biasing; in particular, smaller males and those with a larger genital spicule achieve higher sperm precedence when mating first. My results provide empirical support for the hypothesis that evolution of male genital morphology is driven by sexual selection.

Male genitalia morphology

For the past two decades, Eberhard (e.g., 1985; 1993b; 1996; 1997; 2004a,b; Eberhard and Ramirez 2004) has invoked post-copulatory sexual selection as a likely explanation for the complexity and diversity of the genitalia of male insects and other arthropods. However, direct empirical support of this hypothesis has been surprisingly scant given the strong interest in sperm precedence in general (but see Otronen 1998; Arnqvist and Danielsson 1999a; Danielsson and Askenmo 1999; House and Simmons 2003; Rodriquez et al. 2004). My data show that size of the spicule on the genitalia of male oriental beetles is correlated with relative fertilization success, and thus provide

further support that genital morphology of male insects is shaped by sexual selection. Given the relative dearth of similar such explicit tests of the effects of genital morphology on fertilization success, the importance of this phenomenon might be more pervasive than current data suggest. Indeed, Fisherian selection may be particularly compatible with genital structures, which may be less likely than conspicuous ornaments or behavioral displays to incur survival costs (e.g., increased predation risk), perhaps permitting more freedom for "runaway" evolution (Andersson 1994).

I have no data that point to the precise function of the spicule that was found to affect fertilization success in oriental beetle; therefore, I can only speculate. Size of the spicule of the second male did not affect second male sperm precedence, so a role in sperm removal seems unlikely. Because it is located at the base of the aedeagus and does not deeply penetrate the female during copulation (personal observation), the spicule is unlikely to directly facilitate the transfer of sperm. Male insects are generally unable to directly access sites of sperm storage (but see Rodriguez et al. 2004) and fertilization, and, because insect sperm are typically not motile, the ejaculate must be mobilized by the female (Eberhard 1996; Simmons 2001). In oriental beetle, the male might induce mobilization of his sperm (or perhaps other responses related to fertilization) by providing stimulation to the female via his genitalia. If this is the case, a larger spicule might contribute to more male-female genital contact and provide more effective stimulation. It is also possible that a larger spicule contributes to better leverage or stability to facilitate deeper penetration or more effective thrusting of the endophallus. Indeed, examination of flash-frozen pairs of beetles in copula (E.J.W., unpublished data) revealed that the spicule is positioned just inside the vagina, where it

"hooks" onto the female's last abdominal segment. However, it is unclear how a larger ventral area of the spicule would contribute to more effective clasping. It should be noted that size of the spicule and the endophallus were positively correlated (Table 4.1), so it may be that the effect of spicule size on relative paternity was mediated in part or even largely by size of the endophallus. A larger endophallus might also facilitate more effective stimulation and/or deposition of sperm closer to storage or fertilization sites.

Eberhard et al. (1998) suggested that males with intermediate genital morphologies should have greater fertilization success on average, because the level of stimulation by the male genitalia that is perceived internally by females should be partly influenced by the size and genital morphology of females (thus, males of intermediate genital morphology would be "compatible" with more females). Relatedness or biochemical compatibility have been found to affect sperm precedence in some species (Clark et al. 1995; Wilson et al. 1997), and it is conceivable that morphological compatibility of the genitalia could be important as well (see Arnqvist and Danielsson 1999a), which would be expected to favor intermediate male genitalia size. However, my results are similar to those of House and Simmons (2003) in that the genital spicule of oriental beetle appears to be under directional, rather than stabilizing selection (see Figure 4.3). Nevertheless, it is still possible—as suggested by House and Simmons (2003)—that variation in genital morphology is balanced by natural selection, which should favor genital morphology that remains compatible with the basic mechanics of coupling and sperm transfer. Future studies should include measurements of female genitalia morphology to test for a morphological compatibility effect on sperm precedence patterns.

The mechanism by which males with certain morphological features of the genitalia achieve greater relative paternity remains largely unknown in other species, as well. In addition to assessing the role of genitalia morphology in sperm precedence across more groups of insects, future work should be aimed at understanding the precise function(s) of genital sclerites that are associated with relative fertilization success. While mediation of cryptic female choice via specialized stimulation is an intriguing possible function of complex male genitalia, relatively straightforward processes, including facilitation of the depositing of sperm closer to storage or fertilization sites (which is still within the scope of cryptic female choice) are possible as well. Distinguishing between sperm competition and cryptic female choice has proven challenging, and complex interactions between these non-mutually exclusive processes may be expected (Eberhard 2000). Moreover, there is reason to expect that the major non-sexual selection models (the lock-and-key and pleiotropy hypotheses) are not completely without merit. For example, evolutionary changes in genital morphology might contribute to reproductive isolation early in the speciation process (Shapiro and Porter 1989), and pleiotropy has been found to contribute to evolution of genital morphology in a water strider (Arnqvist and Thornhill 1998).

Male body size

My finding that smaller males—when mating first—achieved greater relative paternity is puzzling, given that this result is at odds with much of the current theory on pre- and post-copulatory sexual selection (see Introduction). One possible explanation is that there is a trade-off between male body size and the size (and productivity) of the

testes or accessory reproductive glands. The existence of such a trade-off seems possible for oriental beetle since adult males feed very little, if at all (Friend 1929; Hallock 1933), and apparently all of the biochemical resources available for ejaculate production must be present within the male at eclosion. I am aware of no other study that has demonstrated such a trade-off; on the contrary, the general correlation in insects between male body size and relative fertilization success (see Introduction) suggests an underlying correlation between body size and ejaculate production. Nevertheless, the positive correlation between male body size and sperm precedence is not universal across insects, and in some moths, for example, small males are able to produce spermatophores that are just as nutrient-rich as those produced by larger males (Bissoondath and Wiklund 1996).

It is also possible that males use different mating strategies depending on their size. For example, even if larger males produce greater quantities of sperm or accessory gland products, if they also generally achieve higher mating frequencies (perhaps because they out-compete smaller males for access to females), they might be selected to invest less into each mate. Conversely, if smaller males are less likely to mate more than once, they could be selected to invest more reproductive effort into any given mating, which could give them an advantage in sperm competition with larger males. In other words, larger males may transfer fewer seminal products than they are otherwise able on their first mating in "anticipation" of future mating events. Evidence for such a strategy has recently been found in black-horned tree crickets (Bussière et al. 2005), where larger males (which are preferred by females) decrease the size of nuptial

gifts in response to the perception of more future mating opportunities. Whether a similar situation exists in oriental beetle has yet to be tested.

A similar scenario in which males exhibit size-dependent investment in ejaculates has been proposed for water striders (Arnqvist and Danielsson 1999b; Danielsson 2001), in which smaller males might on average achieve higher relative paternity in any given mating *because* they exhibit lower mating frequency than larger males—and are, thus, less likely to be depleted of ejaculate reserves. Because each male in my experiment mated only once (and was not depleted from multiple mating), this line of reasoning cannot directly apply; but it remains to be tested whether larger male oriental beetles are able to judiciously dole out ejaculates among their mates.

Though never examined directly, several points bear upon a possible relationship in oriental beetle between male body size and mating frequency in the field. First, in systems that feature scramble competition polygyny (Nahrung and Allen 2004) or in which agility may be more important than brute force in mate acquisition (Crompton et al. 2003), smaller males might experience an advantage in pre-copulatory selection (Steele and Partridge 1988) or realize higher mating success in the field (Neems et al. 1998; LeBas et al. 2004; Nahrung and Allen 2004)—especially when competing in three-dimensional habitats (e.g., air, water) where males are unable to take advantage of larger body size by using substrate as leverage. Oriental beetle does exhibit scramble competition polygyny; however, given that mating and orientation to females both occur in close association with the ground surface (Facundo et al. 1999a) and intrasexual competition for females may be strong (Bianchi 1935; Facundo 1997), it seems unlikely that smaller rather than larger size would confer an advantage to males

in acquisition of mates. However, Facundo et al. (1999a) found that difference in male size did not influence the ability of single males to overtake (or resist takeover attempts of) pairs in copula in the lab (the authors reported only six successful takeovers, but did not report how many of the 21 trials featured a takeover attempt). Second, length of the right antennal club of males used in the double mating experiment was correlated with body mass ($F_{1,113} = 17.1$, $P < 0.0001$, $r^2 = 0.132$); larger antennae might confer an advantage in detecting female sex pheromone and contact pheromone (Jiménez-Pérez and Wang 2004b) and could contribute to higher mating frequency in the field for larger males. Finally, my data from a subsequent study looking at the effect of male mating history on female reproductive output (Chapter 5) suggest that male body size is indeed positively correlated with lifetime reproductive success. This pattern is consistent with the idea that larger males are better adapted to multiple mating and partition resources more evenly among their mates. Clearly, more rigorous tests will be needed to elucidate the importance of male size to both mating frequency in the field as well as lifetime reproductive success in polyandrous situations.

Copula duration

The impact of copula duration on paternity may be difficult to recognize if larger males tend to mate for shorter durations but transfer sperm and/or accessory gland products at a faster rate (see Simmons and Parker 1992; Sih et al. 2002); conversely, smaller males might copulate longer to compensate for slower transfer of seminal products (Vermette and Fairbairn 2002). For oriental beetle, copula duration of the first male tended to decline with increasing male mass, but the relationship was not

significant (FS + SF treatments: $F_{1,32} = -2.66$, $P = 0.113$, $r^2 = 0.079$); results were similar for copula duration of the second male ($F_{1,32} = 2.23$, $P = 0.146$, $r^2 = 0.067$). Even if larger males were slightly more efficient at transfer of seminal products, it may not be surprising that male body size did not strongly impact copula duration. I found males to be refractory to mating for at least two hours after copulation (E.J.W., unpublished data), suggesting that males in the field may mate only once per evening at most; thus, potential costs from missed mating opportunities may not pose strong selection on males to limit copula duration, as has been suggested, for example, in the yellow dung fly *Scatophaga stercoraria* (Parker and Simmons 1994). However, potential for mating opportunities on later days might still contribute to optimal copula duration in oriental beetle if time spent in copula is correlated with the volume of ejaculate transferred. In any event, inclusion of male mass in the regression model for P_2 should have helped to compensate for any weak effects of size on mating time.

My results provide suggestive evidence that increased relative paternity may have been mediated by longer copula duration, especially for the second male to mate (Figure 4.4). Longer copulation for the second male might have facilitated the transfer of a larger ejaculate to better compete with previous males or perhaps manipulation of the previous male's ejaculate. While well documented in the Odonata (Simmons 2001), sperm removal has been found in but a handful of non-odonates, including a longicorn beetle (Yokoi 1990) and *Tribolium castaneum* (Haubruge et al. 1999). For oriental beetle, it is possible that the patches of sclerotized spines at the base of the endophallus (see Figure 4.1) function in removal of rival sperm. The fact that the effect of copula duration of the second male differed between treatments (see Figure 4.4-B) might

reflect the transfer of fewer sperm by irradiated males, making any sperm removal effects less clear in the SF treatment. Any weak effect on relative paternity from longer copula duration of the first male (see Figure 4.4-A) might have been mediated by the production of a stronger signal to generate refractory behavior in the female and/or stimulation to use the first male's sperm. I caution, however, that I have no direct evidence to clarify any function of longer mating duration.

Copula duration may be expected to be longer for a male mating with a non-virgin female if the male adjusts his mating strategy based on the increased risk of sperm competition (for example, to manipulate the ejaculate of any prior males) and/or it takes the male longer to achieve complete intromission (see Eberhard 2002 and Tallamy et al. 2002) because the female is reluctant to remate. It is possible that the latter explanation contributes to increased copula duration of second males, which was found only for FF treatments (Table 4.4). Examination of flash-frozen pairs of beetles in copula at either 2 or 10 minutes into copulation for first and second males (E.J.W., unpublished data) showed that—while all first males and most second males achieved full eversion of the endophallus, whether examined after 2 or 10 minutes after intromission—one male had still not everted his endophallus even after 10 minutes of copulation. Thus, longer copula duration of second males may be partly explained by difficulty in achieving full inflation of the endophallus, and it is even possible that some males never fully everted the endophallus. The fact that copula duration for FS and SF treatments did not differ between the first and second male (Table 4.4) might be explained by some reduction in the mating ability of sterilized males. If sterilized males are generally unable to mate for the same length of time as un-sterilized males (my

results suggest, but do not compellingly demonstrate this), then we might expect no difference in copula duration between males for the FS treatment. Alternatively, if sterilized males in the SF treatment were less effective at inducing refractory behavior in females, second (fertile) males might not have needed to adjust their copula duration and/or females may have exhibited less resistance to intromission; my results suggest that female resistance to the second male was indeed weaker in SF treatments (Table 4.4).

First versus second male advantage

My results are unusual among sperm precedence studies in that morphological traits only of the first male were found to impact paternity. In a preponderance of studies of sperm precedence in insects using two males, variance in P_2 has been attributed to characters of the second male alone (Simmons 2001). That variance in relative paternity was only weakly related to a character of the second male (copula duration) suggests that in oriental beetle the ability of males to assess female mating status and adjust mating behavior accordingly may be limited (cf. Wedell and Cook 1999). Male mate choice based on female reproductive status is common in insects (Carazo et al. 2004 and references therein); thus, even if male oriental beetle do not exhibit mate choice it seems likely that they would at least be able to assess whether they are mating with a non-virgin female [perhaps based on changes in titer or composition of contact olfactory cues (Polerstock et al. 2002) or even increased female resistance] and adjust their mating strategy to better compete with the ejaculate of a

prior male. Possible negative effects of the sterilization process could also account for the apparent lack of an adjustment when mating with non-virgin females (see above).

Clearly, the possibility that I failed to measure one or more traits that are important for second male sperm precedence in oriental beetle cannot be discounted; however, it is interesting that male size—a parameter known to influence second male paternity across many insect taxa (Simmons 2001)—was important in influencing paternity for first, but not second males. Both "offensive" (contributing to preemption of the sperm of prior males) and "defensive" (contributing to avoidance of sperm preemption by subsequent males) functions in paternity biasing have been found in the genitalia of water striders (Anrqvist and Danielsson 1999a) and dung beetles (House and Simmons 2003). In both of these cases, the offensive and defensive traits were independent, which further points to the possibility that some trait in males—perhaps another aspect of genital morphology—was overlooked in my study. My simple, two-dimensional measurements of the genitalia undoubtedly underestimated the overall variation in morphology; however, Arnqvist and Thornhill (1998) found that—when comparing patterns of variation in general and genital morphology—simple linear measures yielded essentially the same result as more complex measures of shape. Indeed, my preliminary analyses used simple linear measures of genital morphology and yielded no real differences in the results (data not shown). It is also worth noting that the amount of variance in P_2 that my model explained was comparable with other similar studies.

The fact that traits of the first male had primacy in influencing relative paternity may be consistent with the idea proposed above that smaller males might invest more

into any given copulation, while larger males spread their reproductive investment over more mates. It is possible that the traits that contribute to male reproductive success—which could include stimulation of ovulation, oviposition, mobilization and storage of sperm, and/or female refractory behavior—also function to make a male good at "resistance" of any subsequent competitors for the female's ova. My data show an interesting pattern that bears upon this issue. When considering only the FS treatment, fecundity tended to decline with increasing P_2 value (Figure 4.5; full model including first male mass and spicule size: $F_{3,15} = 5.04$, $P = 0.017$, $r^2 = 0.557$; partial regression slope: $t = -0.52$, $P = 0.615$); removal from the analysis of one data point that was an outlier with respect to the overall effect of copula duration on P_2 (and the only case in which the female laid no fertile eggs, which calls into question the fertility of this female and/or the first male) rendered the effect significant (full model: $F_{3,14} = 4.32$, $P = 0.030$, $r^2 = 0.541$; partial regression slope: $t = -2.25$, $P = 0.046$). Thus, the greater the relative paternity of the first male, the more eggs the female laid. It is, therefore, conceivable that male traits that enhance the reproductive output of his mate also contribute to resisting preemption of that male's sperm. SF treatments did not show the same pattern (full model including first male mass and spicule size: $F_{3,16} = 0.87$, $P = 0.480$, $r^2 = 0.168$; partial regression slope: $t = 0.33$, $P = 0.746$), possibly because sterilization reduced the production or transfer of fecundity-enhancing seminal components. If traits that enhance a male's relative fertilization success overlap with those that enhance his mate's reproductive output, then characters of the first male may be expected to be particularly important in influencing paternity, especially if females that are refractory toward additional matings are also less likely to mobilize the sperm

of any subsequent males that induce copulation. Moreover, if polyandry is relatively uncommon in the field (perhaps because males are generally able to successfully escort females back into the soil before other males can mate with them), then selection on means to preempt the sperm of previous mates could be relatively weak. Estimation of the degree of polyandry in the field as well as study of the mechanism by which certain males resist preemption of their ejaculate will be necessary to further elucidate these issues.

I am aware of a scant few examples in insects in which traits of the first male to mate have primacy in influencing relative paternity. In *Drosophila pseudoobscura*, males of certain karyotypes were better able to avoid displacement of their sperm by subsequent males; karyotype of the second male and of the female had no effect on relative paternity (Turner and Anderson 1984). In the southern green stink bug, P_2 was lower when the first male was larger (McLain 1985) or mated longer (McLain 1980). Interestingly, larger males also yielded greater fertility (McLain 1980), and females were more likely to oviposit before remating when the first mating was of sufficient duration (McLain 1981). These patterns in the southern green stink bug are consistent with my suggestion for oriental beetle (above) that male traits that enhance reproductive output in their mate might also contribute to higher relative paternity when such males mate first. It is noteworthy that increased rate of oviposition after mating with certain males is the context in which the term *cryptic female choice* was first used (Thornhill 1983).

Conclusions

The results reported here provide further experimental evidence that the genitalia of male insects evolve by post-mating sexual selection. Given that female oriental beetle lack direct pre-copulatory mate choice and males do not exhibit overt pre-copulatory courtship behavior, I predicted that post-copulatory sexual selection would be strong in this species. Certainly strong pre-copulatory mate choice does not preclude post-copulatory choice; however, it is possible that the forces of pre- and post-copulatory selection are counteractive (e.g., Danielsson 2001; Sih et al. 2002), and post-mating mechanisms of paternity biasing might be particularly evident in species with limited pre-mating sexual selection. My results also challenge the traditional view of the general reproductive advantage of large size in males. More work will be needed to clarify (1) why smaller males achieved greater relative paternity when mating first, (2) whether male size is related to mating frequency in the field, and (3) the precise role that certain genital morphologies play in relative fertilization success in oriental beetle.

Table 4.1. Correlation matrix for the morphological measures of males. Displayed are Pearson correlation coefficients, with P-values in parentheses below each coefficient. See Figure 4.1 for pictures of genital structures.

	Mass	|FA| hind tibiae	Lateral lobe	Spicule	Spinose patch
Mass	—				
|FA| hind tibiae	-0.054 (0.670)	—			
Lateral lobe	0.385 (0.001)	-0.380 (0.002)	—		
Spicule	0.192 (0.122)	0.063 (0.618)	0.177 (0.155)	—	
Spinose patch	0.144 (0.250)	-0.116 (0.356)	0.280 (0.023)	0.032 (0.799)	—
Endophallus	0.086 (0.494)	-0.028 (0.824)	-0.133 (0.289)	0.254 (0.039)	-0.101 (0.422)

Table 4.2. Egg fertility and P_2 values (untransformed) across treatments.

		Egg fertility		P_2	
Treatment	N	Mean ± SD	Range	Mean ± SD	Range
FF	19	94.7 ± 7.0	75.7–100	—	—
SS	6	4.6 ± 11.3	0–27.8	—	—
FS	16	56.8 ± 27.4	0–95.7	0.43 ± 0.30	0.004–1.05
SF	17	69.8 ± 36.0	0–100	0.71 ± 0.40	-0.05–1.04
FS + SF	33	63.5 ± 32.3	0–100	0.58 ± 0.38	-0.05–1.05

Table 4.3. ANOVA of the portion of offspring sired by the second male to mate (P_2, arcsine transformed) as a function of the parameters retained in the final regression model derived by hierarchical partitioning (using Schwarz Bayesian Information Criterion).

Source of variation	F	P	r^2	Estimate	SE	t	P
Model	6.32	0.002	0.395				
treatment (FS or SF)				-0.31	0.14	-2.16	0.039
mass (first male)				0.05	0.02	2.45	0.021
spicule size (first male)				-28.6	10.0	-2.86	0.008

Table 4.4. Comparisons between the first and second male to mate, separated by treatment. Data are presented as mean ± SE. Means were compared by paired t-tests or Wilcoxon signed-rank tests.

"Foreplay" time	FS + SF	FS	SF	FF	SS
first male	0.38 ± 0.04	0.32 ± 0.04	0.42 ± 0.06	0.47 ± 0.12	0.74 ± 0.23
second male	2.86 ± 0.66	1.60 ± 0.25	3.90 ± 1.1	1.68 ± 0.38	0.64 ± 0.19
Test statistic	$t_{30} = -9.54$	$t_{13} = -8.67$	$t_{16} = -6.13$	$t_{17} = -5.55$	$z_5 = 1.58$
P-value	<0.0001	<0.0001	<0.0001	<0.0001	0.115

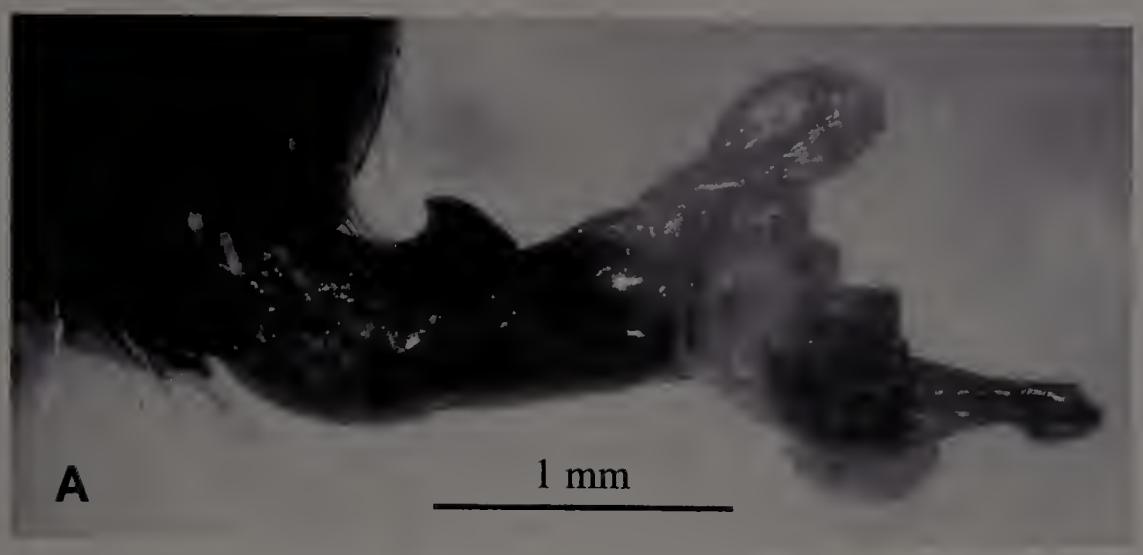
Copula duration	FS + SF	FS	SF	FF	SS
first male	27.8 ± 1.3	29.9 ± 1.8	25.9 ± 1.9	26.3 ± 1.4	26.3 ± 3.7
second male	29.1 ± 1.3	28.9 ± 1.4	29.3 ± 2.1	32.2 ± 1.6	33.2 ± 3.0
Test statistic	$t_{32} = -0.667$	$t_{15} = 0.37$	$t_{16} = -2.81$	$t_{18} = -2.81$	$z_5 = 0.31$
P-value	0.509	0.717	0.251	0.012	0.753

Resistance time	FS + SF	FS	SF	FF	SS
first male	1.12 ± 0.31	1.26 ± 0.52	0.99 ± 0.36	1.02 ± 0.56	0.54 ± 0.28
second male	3.49 ± 0.92	4.05 ± 1.56	2.96 ± 1.05	3.00 ± 0.80	4.18 ± 1.51
Test statistic	$z_{32} = 2.83$	$z_{15} = 2.27$	$z_{16} = 1.82$	$z_{18} = 2.15$	$z_5 = 2.20$
P-value	0.005	0.023	0.069	0.032	0.028

Male mass	FS + SF	FS	SF	FF	SS
first male	20.4 ± 0.66	19.2 ± 0.98	21.5 ± 0.81	20.6 ± 1.13	19.0 ± 1.4
second male	20.3 ± 0.63	21.3 ± 0.93	19.3 ± 0.83	19.5 ± 1.03	18.6 ± 1.8
Test statistic	$t_{32} = 0.13$	$t_{15} = -1.85$	$t_{16} = 2.21$	$t_{18} = 0.76$	$z_5 = 0.41$
P-value	0.901	0.084	0.042	0.460	0.686

Spicule size	FS + SF	FS	SF	FF	SS
first male	0.040 ± .001	0.039 ± .002	0.041 ± .002	—	—
second male	0.043 ± .001	0.043 ± .002	0.043 ± .001	—	—
Test statistic	$t_{32} = -1.64$	$t_{15} = -1.42$	$t_{16} = -0.85$	—	—
P-value	0.112	0.177	0.409	—	—

"Foreplay" values were log-transformed for analysis; untransformed values are shown here. See Methods for explanations of each parameter measured. Male mass was measured in mg and spicule size in mm^2; all times were measured in minutes.



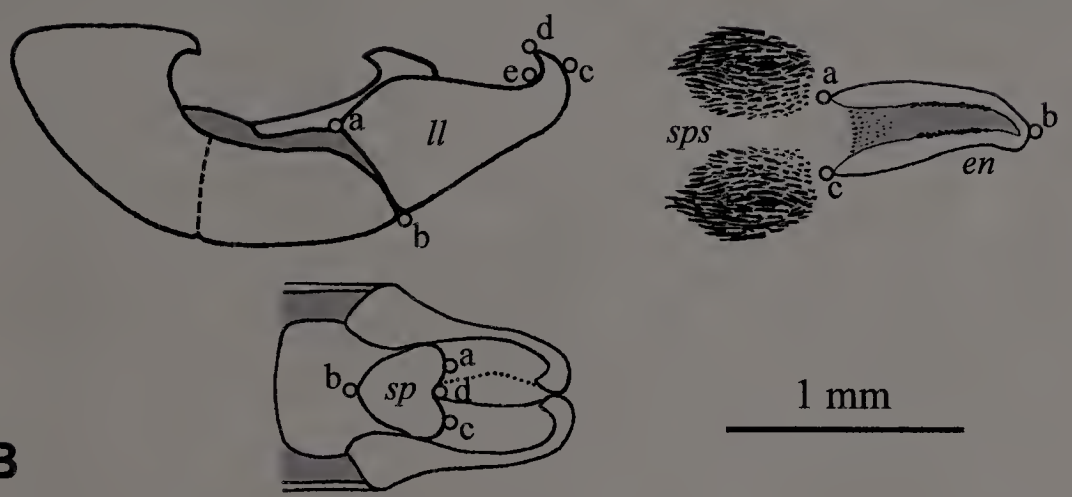


Figure 4.1. Genitalia of male oriental beetle. (A) Photograph of everted aedeagus with partially inflated endophallus, obtained by dissecting a flash-frozen pair of beetles in copula. (B) Schematic of structures of the genitalia that were measured in this study. Morphology of the lateral lobe (*ll*, lateral aspect), spicule (*sp*, ventral aspect), and endophallus (*en*, ventral aspect) were characterized by measuring the area bound by readily distinguishable landmarks (labeled *a*, *b*, *c*, *d*, or *e*). For the two spinose patches (*sps*, ventral aspect), I traced the edges of both structures, calculated the area of each, and used the mean of the two areas as the estimate of spinose patch size. Genitalia terminology follows Friend (1929). Shaded areas represent less sclerotized tissue.

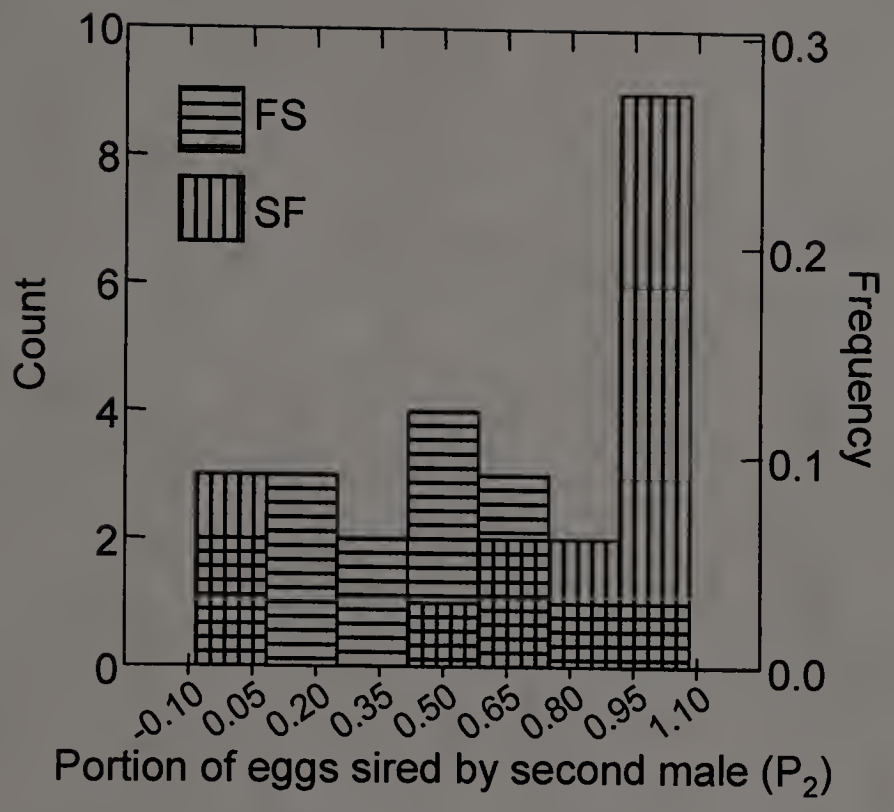


Figure 4.2. Last male fertilization success (P_2, untransformed values) separated by treatment.

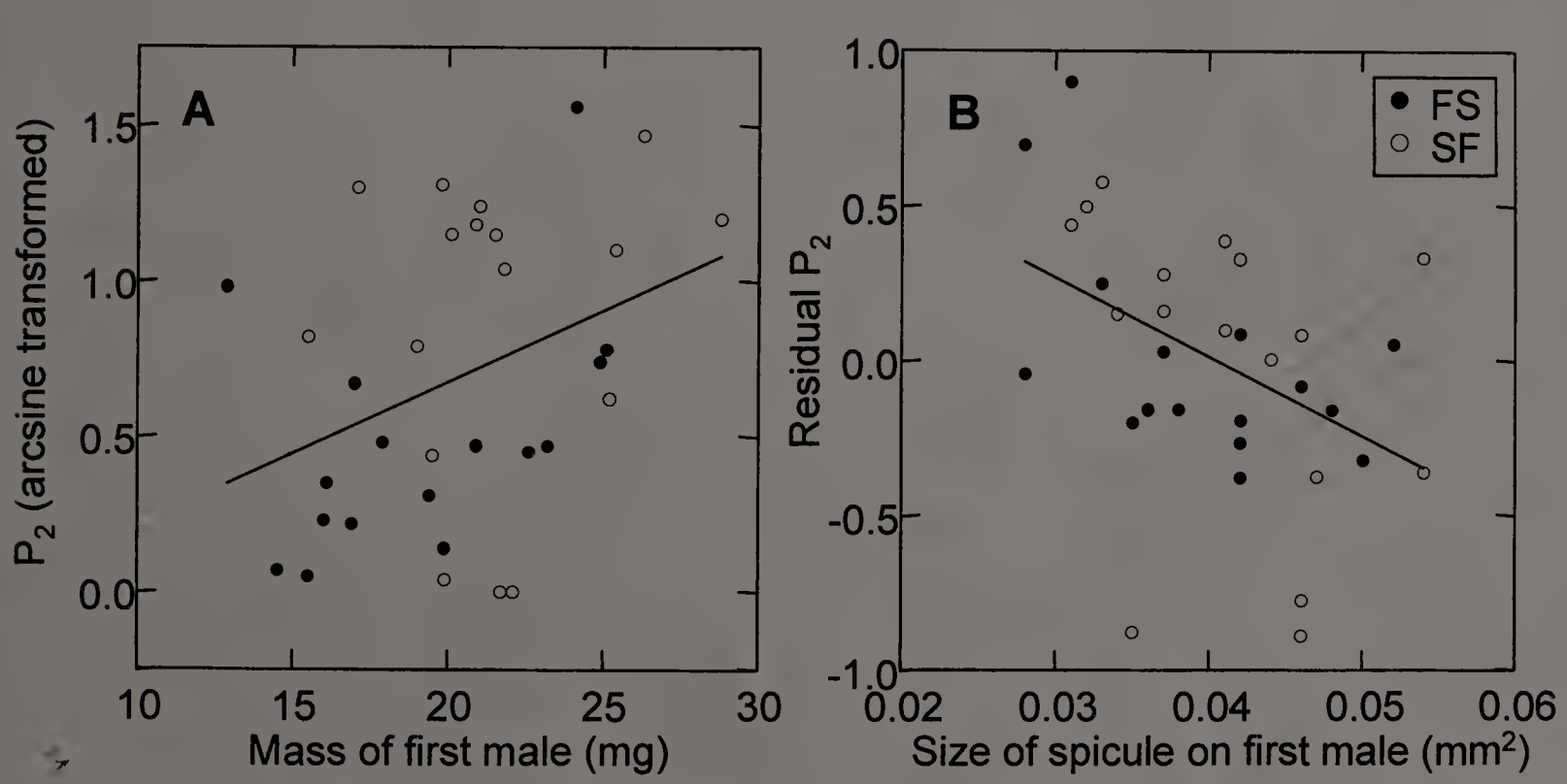


Figure 4.3. Effect of mass of the first male to mate on last male fertilization success (P_2) (A) and effect of size of the genital spicule of the first male to mate on last male fertilization success, accounting for effects of male mass and treatment (B). Plotted in (B) is the relationship between spicule size and the deviation from expected P_2 due to male mass and treatment [i.e. the residuals following the regression of P_2 (acrsine transformed) on mass of the first male and treatment].

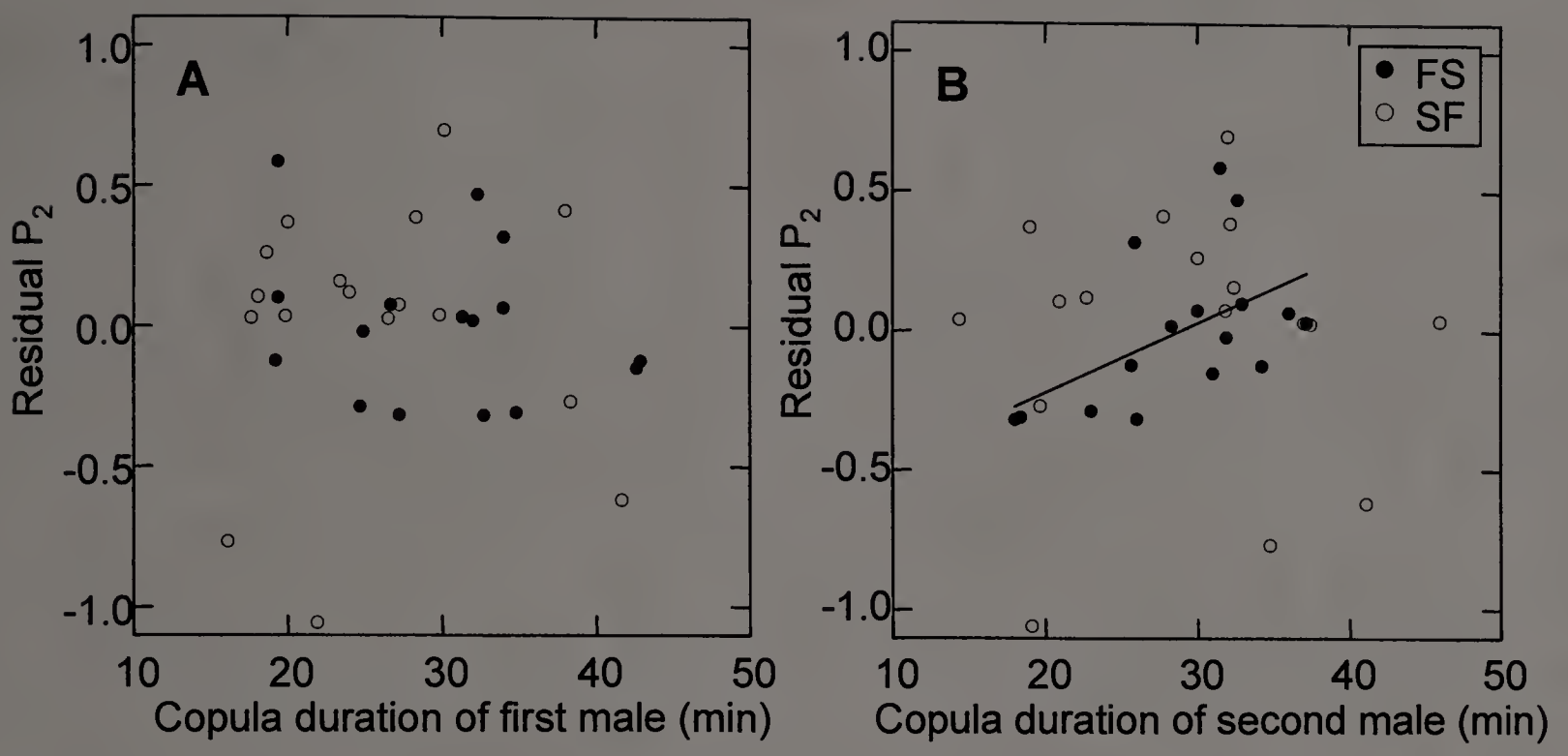


Figure 4.4. Effect of copula duration of the first male (A) and second male (B) to mate on last male fertilization success (P_2, arcsine transformed), accounting for effects of male mass, spicule size, and treatment (see Figure 4.3). Plotted are the relationships between copula duration and the deviation from expected P_2 due to male mass, spicule size, and treatment [i.e. the residuals following the regression of P_2 (arcsine transformed) on mass and spicule size of the first male and treatment]. The trend line in (B) applies only to the FS data points.

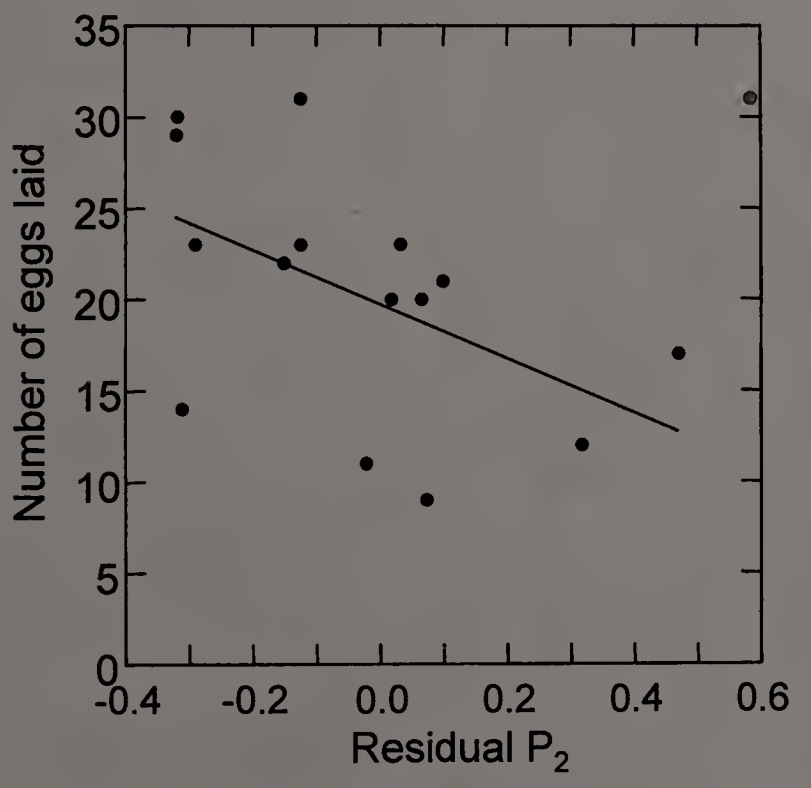


Figure 4.5. Relationship between fecundity and last male fertilization success (P_2) for FS treatment, accounting for effects of male mass and spicule size (see Figure 4.3). Plotted is the relationship between fecundity and the deviation from expected P_2 due to male mass and spicule size [i.e. the residuals following the regression of P_2 (arcsine transformed) on mass and spicule size of the first male]. The trend line excludes the one grayed point that was an outlier with respect to the overall pattern.

CHAPTER 5

THE ROLES OF MALE SIZE AND MATING HISTORY IN THE REPRODUCTIVE OUTPUT OF FEMALE ORIENTAL BEETLE

Abstract

Although the cost of ejaculates has historically been considered trivial, we now appreciate that male reproductive potential may be limited by both access to females as well as ejaculate production. Because males often become depleted of seminal products with successive matings, they may be selected to optimally partition their ejaculates with regard to the number of females they can mate. Male size may factor prominently into these predictions. For example, if larger males are better at acquiring mates (as is generally the case across animals), they may be expected to spread their reproductive effort across more females; in contrast, smaller males may have lower mating frequencies and, thus, be selected to invest relatively more of their ejaculate reserves into any given copulation. I examined how male size and mating history in oriental beetle impacted the reproductive output of females by mating males to three virgin females—one on each of three consecutive days. My previous work with this species showed that relative paternity of the first male to mate a twice-mated female was inversely related to male size. I show here that reproductive output of males is reduced in successive matings only for smaller males. These results suggest that after their first mating, smaller males must either compete using a lower quality ejaculate or submit to a longer refractory period to replenish ejaculate reserves. Thus, the sexually selected advantage of smaller males in their first mating is apparently balanced by lower lifetime reproductive potential relative to larger males.

Introduction

Historically, the cost of ejaculates has been considered negligible (Thornhill and Alcock 1983), and the lifetime reproductive potential of males has been thought to be limited only by access to females (Bateman 1948). Although the difference in gamete size between the sexes is obvious, Dewsbury (1982) pointed out that the cost of the ejaculate is much greater than that of individual spermatozoa. The ejaculate may contain (1) much more sperm than are stored by the female (Parker 1970; Gilbert 1981; Eady 1995), (2) a variety of accessory gland secretions that may influence female responses, including ovulation, oviposition, and refractory behavior (Simmons 2001), and (3) nutrients that can be used by the female to increase her reproductive output (Fox et al. 1995b; Karlsson 1995; see also Eberhard 1996 and Simmons 2001 for criticism of possible nutritive effects). When females mate multiply, size and quality of the ejaculates they receive can be important in influencing relative fertilization success among males (e.g., LaMunyon and Eisner 1994; Eady 1995). Thus, sperm competition and cryptic female choice likely impose selection against males that invest minimally in their ejaculates. This may in turn limit the degree to which males can be promiscuous but still achieve high per-female reproductive output and successfully compete among other males for the fertilization of ova.

Male insects generally become depleted of ejaculate reserves with successive matings, and depleted males elicit weaker refractory behavior in their mates and/or yield lower fecundity compared with virgin males or males that are allowed to recover from mating activities (reviewed by Simmons 2001). Much of the research regarding

the effect of male mating history on female reproductive output has been conducted on species of Lepidoptera (reviewed by Torres-Vila and Jennions 2005), in which males transfer large, nutrient-rich spermatophores. However, similar results have been found for insect species in which the ejaculate may be a less substantial investment (e.g., a planthopper, Heady 1993; a blow fly, Smith et al. 1990; and *Drosophila melanogaster*, Prowse and Partridge 1997). Considering ejaculate size may be a major factor in determining relative paternity (see above), it is not surprising that mating history can also negatively impact a male's fertilization success when females are mated multiply (Holmes 1974; Lewis 2004; Oberhauser et al., unpublished manuscript, cited in Simmons 2001; cf. Wedell and Cook 1999).

Male mating frequency often varies among individuals within species, with larger males generally out-competing smaller males for access to mates (Andersson 1994). If mating frequency of males is correlated positively with body size, then larger males might often be at a disadvantage when their sperm are competing for fertilizations within females that have mated other males with more limited mating histories (Arnqvist and Danielsson 1999b). Because of the potentially high cost of seminal components, males should be selected to optimally partition their ejaculates with regard to the number of females with which they mate (Dewsbury 1982). Thus, it is possible that males employ different mating strategies according to differences in their potential to remate. For example, males that are better competitors for mates (say, larger males) might be selected to partition relatively small portions of their ejaculate production among more mates (e.g., Bussière et al. 2005). Conversely, if smaller males are at a competitive disadvantage in achieving matings, they should not conserve

resources for unlikely future matings, rather they should transfer a larger portion of their available ejaculate as an adaptive response to remating intervals that are relatively long.

My previous work with oriental beetle, *Anomala orientalis* (Waterhouse) (Coleoptera: Scarabaeidae), showed that in twice-mated females, relative male fertilization success was influenced in part by size of the first male; surprisingly, smaller males achieved higher relative paternity (Chapter 4). Because I found evidence of a relationship between fecundity and relative paternity of the first male to mate, relative fertilization success might be linked with ejaculate investment, perhaps by transfer of oogenesis and/or oviposition stimulants (see Chapter 4). I hypothesized that males exhibit size-based mating strategies related to differences in their potential to remate (see above). A key prediction of this hypothesis—if components of the ejaculate that enhance fecundity also bias paternity—is that smaller males should yield greater fecundity in their mates for their first mating relative to larger males for their first mating. Moreover, when given the opportunity to mate multiply, smaller males should become depleted of ejaculate reserves faster, and larger males should achieve greater lifetime reproductive fitness.

I tested the hypothesis that male size in oriental beetle is positively correlated with lifetime reproductive potential by mating males to multiple virgin females over consecutive days. I predicted that smaller males would become depleted of ejaculate faster than larger males, which would be reflected as a decline in fecundity of their mates. This work also has implications for pest management—specifically mating disruption—because the capacity of males to remate can potentially confound efforts to reduce the local population by disruption of communication between the sexes.

However, if males elicit lower fecundity in females with successive matings and/or exhibit long refractory periods following mating, then the capacity for a small portion of the males within a population to fertilize a large portion of the females may be limited.

Methods

Collection and rearing of beetles

I collected third instar grubs from turf at Bay Pointe Country Club golf course, Onset, MA on 18 May 2004 and on 25 October 2004. All grubs were collected into plastic containers with soil, then taken back to the lab where they were placed individually into lidded 30 mL clear plastic cups with prepared soil. The soil mixture was made by mixing two parts screened sand with one part screened peat moss (1.18 mm sieve). I sterilized the soil in an autoclave (123˚C, 1 kg/cm^2 for 60 min), allowed it to cool, and then added sterilized, deionized water to moisten the mixture to about 12% water by weight. A heavy pinch of grass seed (Pennington® mix comprised of 63% shining star perennial ryegrass, 15% boreal red fescue, 9% kenblue Kentucky bluegrass, 9% blue bonnet Kentucky bluegrass, 2% inert matter, 1.9% other crop seed, and 0.1% weed seeds) was placed on top of the soil in each cup before closing to provide food for the grubs to continue development. I placed cups on 30-cup capacity clear plastic trays and placed each tray into a clear 9.46 liter plastic, re-sealable bag with a damp sponge to reduce moisture loss from cups. Grubs were held in a rearing chamber on a 16:8 L:D cycle at 10°C to slow development.

About one month before adult beetles were needed, I placed trays in a rearing chamber on a 16:8 L:D cycle at 25°C. I checked each cup weekly for pupae, and

checked pupae daily for adult eclosion in order to establish adult age. Adults were sexed (based on size of antennal lamellae) and held in their rearing cups until needed for mating experiments.

Multiple mating experiment

I mated beetles in the lab under conditions that simulated dusk—the period of peak female sexual receptivity (Facundo et al. 1994) and peak male responsiveness to pheromone (Facundo et al. 1994; E.J.W., unpublished data). I moved beetles to a room held at about 60% humidity and maintained on a 16:8 L:D cycle (as above for rearing chambers), but at 25-27°C during photophase and 21-22°C during scotophase and the last two hours of photophase. Light intensity during photophase was held at ca. 150 lux, except during the first and last two hours of photophase when it was held at 15 lux. I paired beetles within the last two hours of photophase.

To establish mating pairs, I placed a female in a plastic, 30 mL cup with a layer of soil ca. 0.5 cm deep. Females typically begin calling (i.e. raising the abdomen and releasing sex pheromone) within 2 min of being placed on soil under simulated dusk conditions (personal observation). I introduced a male after the female began calling; males promptly mounted calling females in the lab (personal observation).

I mated each male (n = 6 in August 2004; n = 11 in April 2005) to a different virgin female on three consecutive days (n = 51 females). For each mating pair, I recorded the duration of copulation (length of time between intromission and retraction of the aedeagus). After each mating pair separated, I removed the female and transferred her to a clear, plastic oviposition cup (diameter: 12 cm, height: 13 cm) with

a layer of soil ca. 3 cm deep. Two weeks after mating (when most if not all eggs had been laid), I sorted through the soil in oviposition cups and collected all eggs into Petri dishes lined with filter paper moistened with sterilized, deionized water. If the female was still alive, I returned her to the oviposition cup with fresh soil and collected any additional eggs ca. seven days later. I held eggs at least until fertility could be assessed; fertile eggs increase in size, become more spherical, maintain their white color, and just before hatching, the orange-brown mandibles of the developing larva are visible through the chorion (personal observation).

Longevity and morphological measurements

After use in mating experiments in 2004, I froze males for later morphological measurements (see below). In 2005, I transferred males individually to 30 mL plastic cups filled with soil. I also transferred 8-d-old unmated males (n = 20) to plastic cups with soil. I checked each male daily to assess mortality; unmated males and those that had not mated recently generally remained at or near the soil surface, which allowed for minimal disturbance when checking for mortality. After males died, I measured the dry mass of each male to the nearest 0.1 mg. When females were found dead, I dissected them and counted any unlaid eggs remaining in the abdomen; to estimate female size, I measured the length of each female's right elytron using an ocular micrometer.

Data analysis

I first ran analyses using year as a blocking factor; including the blocking factor was found not to reduce MS error, so I pooled data between years to increase power.

To compare fecundity and total egg production (number of eggs laid + number of eggs remaining in the abdomen at death) among the three mates of each male, I used repeated measures ANCOVA with female elytron length as the covariate. Because egg fertility data were not normal (based on Kolmogorov-Smirnov test) and could not be normalized by transformation, I used Friedman's test to compare fertility among treatments. I used repeated measures ANOVA to compare copula duration among the males' three mates. To compute lifetime reproductive output of males, I first corrected for female size by dividing the number of eggs each female laid or the total eggs she produced by her right elytron length. I then summed each set of three values for the two parameters across the mates of each male. To explicitly evaluate the effect of male size on female reproductive output, I performed linear regression analyses using fecundity and total egg production as the dependent variables, with male mass as the main effect. My measure of female size (length of the right elytron) was used as a covariate, because I have found size to be correlated with egg production (Chapter 3). I evaluated the effect of copula duration on reproductive output in a similar manner, using duration of copulation in the model rather than male mass. All data were analyzed using SAS (version 8.2, SAS Institute, Inc.).

Results

Male mating history did not appear to impact female fecundity or total egg production (Table 5.1); these two parameters did not differ among the males' three mates. However, lifetime reproductive output of males (the total number of eggs laid among each male's three mates) was correlated with male mass ($F_{1,16} = 7.07$, $P = 0.018$,

$r^2 = 0.321$); results were similar for total egg production, although the relationship was not significant at $\alpha = 0.05$ ($F_{1,16} = 3.44$, $P = 0.083$, $r^2 = 0.187$). These correlations reflected male size-mediated variance in fecundity and egg production only for each male's second mating (Table 5.2; Figure 5.1); neither fecundity nor egg production was correlated with male size for the first or third matings (Table 5.2; Figure 5.1). The number of eggs that females produced tended to be more strongly dependent on female size than was the number of eggs that females laid (Table 5.2; see also Table 5.4).

Egg fertility did not differ significantly according to male mating history (Table 5.1).

Copula duration did not differ by male mating history (Table 5.1); however, individual males mated for similar lengths of time over successive copulations (Table 5.3; Figure 5.2). Reproductive output was not correlated with copula duration for the first two matings; however, fecundity and total egg production declined with increasing duration of copulation for the third mating (Table 5.4; Figure 5.3).

Adult lifespan did not differ between virgin males and those mated to three females (t-test, $t_{29} = 0.49$, $P = 0.627$).

Discussion

My previous experiments with oriental beetle showed that relative paternity of the first male to mate twice-mated females was inversely related to his size (Chapter 4). The results presented here contribute toward reconciling my previous work with the general positive relationship between male size and fertilization success in insects (Andersson 1994; Simmons 2001). My hypothesis that larger males would achieve

greater lifetime reproductive success when mating multiple females was supported by the results of the present study. Interestingly, size-mediated variance in the lifetime reproductive success of males was due largely, and perhaps entirely, to variance in egg production and fecundity for each male's second mating. Smaller males apparently became partially depleted of ejaculate after just one mating, whereas larger males showed no clear signs of ejaculate depletion across their three matings. It is not surprising that smaller males became depleted of ejaculate reserves faster than larger males; similar results have been found in other species (e.g., Cook 1997; Fox et al. 1995a; Savalli and Fox 1998) and may be generally expected if male size is positively correlated with ejaculate production (e.g., Fox et al. 1995b; Bissoondath and Wiklund 1996; Schlüns et al. 2003). In a species like oriental beetle in which adult males do not feed (Friend 1929; Hallock 1933), all of the resources used for reproduction must be present at adult eclosion, and one would expect larger males to be capable of producing more ejaculate in a given period of time. Curiously, however, smaller males appeared to recover after their second mating, because fecundity did not vary by male size for the third mating. It may be that some physiological change in the reproductive system must take place after a male's first mating before he is able to increase production of ejaculate for any future copulations; Molleman et al. (2004) made a similar suggestion regarding the butterfly *Bicyclus anynana*. In larger males, any such physiological change might be mitigated by larger ejaculate reserves, or might occur more rapidly. Also, it may be that egg production and fecundity associated with the third mating of smaller males rebounded due to an increase in the rate of ejaculate production in response to high mating frequency. However, I am aware of no evidence for such a

phenomenon in insects; in fact, the rate of sperm production in some moths remains more or less constant after mating (Seth et al. 2002 and references therein), but this issue has apparently received little study in insects.

Although male oriental beetle exhibited size-mediated variance in lifetime reproductive success, these results do not necessarily directly translate to how mating history would affect males in polyandrous situations. My previous experiments suggested that relative fertilization success of males might be linked with the transfer of seminal components that enhance fecundity (Chapter 4). However, it remains unknown what accessory gland secretions in oriental beetle might influence relative male fertilization success and reproductive output, or whether there is overlap in paternity-biasing and fecundity-enhancing functions of any such seminal components. Any such overlap in function is apparently incomplete at best given that smaller males did not yield higher fecundity or egg production in their mates for their first mating. Larger males yielded no change in fecundity and egg production of their mates over three copulations, but it is still possible that the ability of their ejaculates to effectively compete among those of other males for the fertilization of ova was reduced. On the other hand, it seems quite likely that smaller males—when ejaculate-depleted after their first mating—would be less effective in post-copulatory competitions for ova. Future work should be aimed at directly examining how sperm precedence of individual males varies over successive copulations when mated first to doubly mated females.

All males in my experiment were mated to virgin females on successive days; however, in the field, smaller males might not be prepared to re-mate until their ejaculate reserves are replenished—perhaps two days after their first mating. Larger

males, on the other hand, appear able to transfer an adequate ejaculate by the day following their first mating. I have some anecdotal evidence suggesting males might not search for mates until their ejaculate reserves are replenished. Males used in my experiment were maintained in individual cups with soil, and all males immediately burrowed under the soil when placed in their cups after mating (personal observation). On days following mating, previously mated males typically were found with their heads protruding out of the soil surface and their antennae splayed. Males generally did not surface until ca. 22-24 h following their first mating, but often surfaced earlier on the days following their second and third mating (personal observation). The timing of male surfacing behavior might be related with when their ejaculate reserves were replenished. Unfortunately, I did not systematically check for male surfacing behavior at regular intervals every day, so I am unable to test whether males that surfaced earlier yielded higher fecundity in their subsequent mates than those that were still under the soil at the time of their scheduled mating. Nevertheless, if larger males have shorter recovery periods after mating (as suggested by my data), they should tend to have higher mating frequencies and, thus, greater lifetime reproductive success than smaller males—even if they experience lower relative paternity than smaller males. Larger males might experience other advantages that would contribute to higher mating frequencies in the field as well (see Chapter 4).

Copula duration in insects often shows considerable within-species variability due to numerous factors, including male and female size and mating history, operational sex ratio, and genotype (Simmons 2001). The tendency for individual males to mate for similar lengths of time with successive copulations is consistent with the idea that male

genotype is a contributing factor in influencing copula duration for oriental beetle. Although mean copula duration was not affected by mating history, the decline in reproductive output with increased copula duration for the third mating suggests that males that tended to copulate longer were becoming depleted of ejaculate. Males that consistently copulate longer might transfer larger volumes of accessory gland secretions in their ejaculates and, therefore, become depleted sooner. Indeed, fecundity for the third mating tended to decline with increasing total time spent in copula for the first two matings ($t = -1.86$, $P = 0.084$, $r^2 = 0.199$), but the pattern was not significant at $\alpha = 0.05$. Mating times that are more or less constant for individual males may represent another set of "strategies" in which males either engage in shorter copulations—which should function to conserve ejaculate reserves for future matings—or longer copulations, which may enhance the competitive abilities of a male's ejaculate. In polyandrous situations, males that mate for shorter durations might suffer lower relative paternity if their partner remates (see Chapter 4).

Lifespan of adults did not differ between sexually experienced males and virgins. Others have cited a decline in male longevity with increased mating experience as evidence of the cost of ejaculate production (Prowse and Partridge 1997; Simmons 2001). Clearly, the fact that males are refractory to remating for some time suggests a cost of the ejaculate in oriental beetle as well, but it may be that ejaculate production and other physiological processes in this species are not interdependent enough to affect longevity. Indeed, I found no evidence for a tradeoff between reproductive output and longevity in females, either (Chapter 3). Greater energy expenditure of virgin males while in their individual cups (e.g., walking and attempting to climb or fly out of cups;

personal observation) might have contributed to a decreased lifespan, while mated males were less active, but presumably used energy reserves to replenish ejaculate.

The results of this experiment also have implications with respect to management of oriental beetle by disruption of semiochemical communication between the sexes. The ability of a small number of males to mate many females could potentially confound attempts at mating disruption. Although reproductive output was only slightly reduced with successive matings and only for smaller males (and to a lesser extent, those that engaged in longer copulations), the capacity of males to remate is limited compared to some other species. For example, the blow fly *Lucilia cuprina* can mate on average ten females over 10 h of continuous access to virgin females (Smith et al. 1990), and the planthopper *Prokelisia dolus* can mate up to six females in less than 1 h (Heady 1993). I found male oriental beetle to be refractory to remating for at least 3 h (unpublished data), and most males can probably remate at most once per day (see above). These findings suggest that the potential of a few males to mate many females and confound management efforts is limited.

Previous studies with oriental beetle have focused largely on management and monitoring. My research demonstrates that numerous fascinating questions regarding the mating behavior, ecology, and evolution of this species remain to be explored. More work will be needed to further clarify the relationships investigated here among male body size, mating history, and reproductive success as well as how these factors affect mating frequency of males in the field, ejaculate composition, and sperm precedence.

Table 5.1. Mean ± SE fecundity, total egg production, egg fertility, and copula duration of females mated with males of different mating history.

Male mating sequence	Fecundity	Total egg production	Egg fertility	Copula duration
First	30.5 ± 4.0	33.6 ± 3.3	92.5 ± 5.8	26.2 ± 2.5
Second	30.4 ± 3.7	36.1 ± 2.3	89.2 ± 6.1	26.1 ± 2.6
Third	31.8 ± 2.8	33.0 ± 2.1	98.6 ± 0.6	28.7 ± 3.1
Test statistic:	0.07	0.83	1.56	1.03
P-value:	0.931	0.456	0.561	0.381

Table 5.2. Regression analyses of fecundity (A) and total egg production (B) as a function of female size (estimated by length of the right elytron) and male mass, separated by mating sequence.

A. Dependent variable: number of eggs laid

Source of variation	F	P	r^2	Estimate	SE	t	P
First mating:							
Model	8.45	0.004	0.547				
Female size				21.7	6.0	3.65	0.003
Male mass				-0.39	1.2	-0.34	0.738
Second mating:							
Model	7.10	0.007	0.504				
Female size				1.28	13.3	0.10	0.924
Male mass				3.50	0.94	3.71	0.002
Third mating:							
Model	0.12	0.886	0.017				
Female size				2.61	6.3	0.41	0.687
Male mass				-0.33	1.0	-0.33	0.750

B. Dependent variable: number of eggs produced

Source of variation	F	P	r^2	Estimate	SE	t	P
First mating:							
Model	5.65	0.016	0.447				
Female size				17.6	5.4	3.27	0.006
Male mass				-1.14	1.0	-1.09	0.293
Second mating:							
Model	17.8	<0.001	0.718				
Female size				19.5	6.4	3.05	0.009
Male mass				2.09	0.45	4.59	<0.001
Third mating:							
Model	1.33	0.297	0.159				
Female size				7.24	4.5	1.62	0.127
Male mass				-0.27	0.71	-0.37	0.713

Table 5.3. Correlation of successive copula durations.

	t	P	r^2	Estimate	SE
First v. second copula duration	3.45	0.004	0.443	0.69	0.20
Second v. third copula duration	3.65	0.002	0.470	0.82	0.22

Table 5.4. Regression analyses of fecundity (A) and total egg production (B) as a function of female size (estimated by length of the right elytron) and copula duration, separated by mating sequence.

A. Dependent variable: number of eggs laid

Source of variation	F	P	r^2	Estimate	SE	t	P
First mating:							
Model	22.5	<0.001	0.776				
Female size				23.4	3.5	6.68	<0.001
Copula duration				-0.42	0.31	-1.37	0.195
Second mating:							
Model	0.15	0.863	0.021				
Female size				8.10	18.7	0.43	0.671
Copula duration				0.10	0.38	0.26	0.799
Third mating:							
Model	13.1	<0.001	0.669				
Female size				12.1	3.0	4.01	0.002
Copula duration				-0.28	0.10	-2.72	0.018

B. Dependent variable: number of eggs produced

Source of variation	F	P	r^2	Estimate	SE	t	P
First mating:							
Model	13.0	<0.001	0.667				
Female size				15.9	3.2	4.93	<0.001
Copula duration				-0.05	0.28	-0.18	0.858
Second mating:							
Model	3.18	0.073	0.312				
Female size				25.1	10.0	2.51	0.025
Copula duration				-0.13	0.20	-0.64	0.533
Third mating:							
Model	13.3	<0.001	0.672				
Female size				12.1	3.0	4.02	0.001
Copula duration				-0.28	0.10	-2.76	0.016

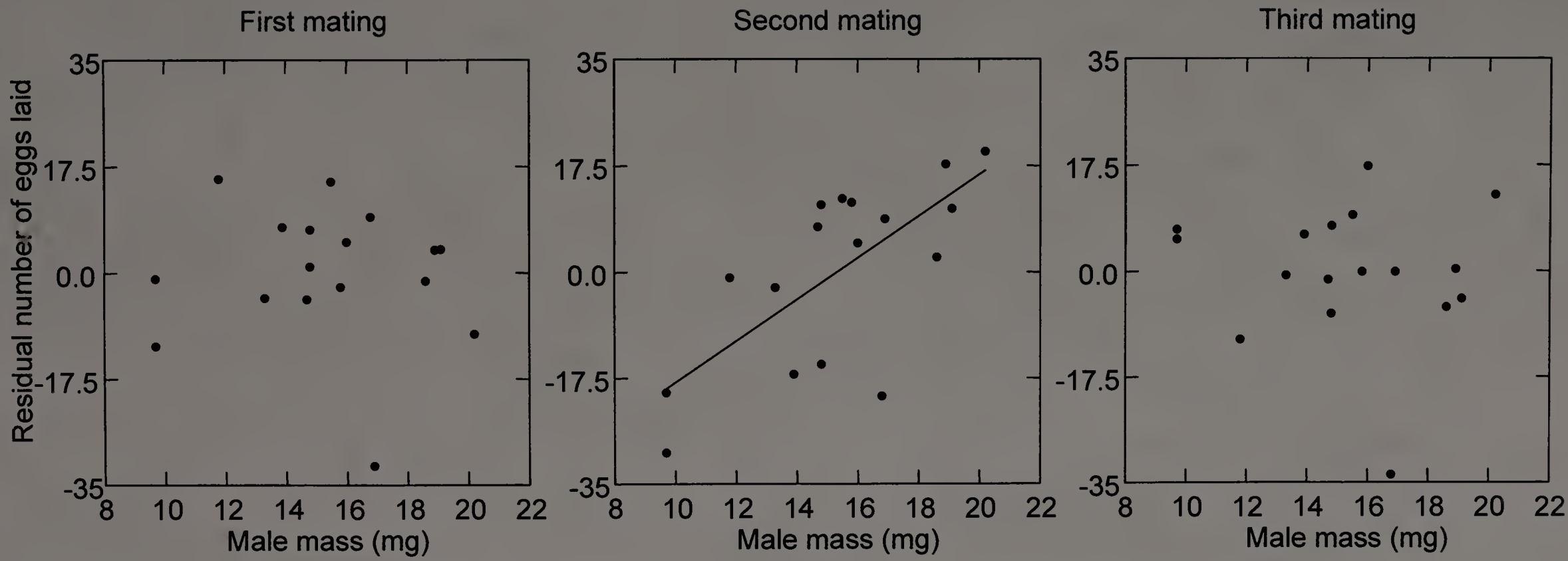


Figure 5.1. Relationship between male size and female reproductive output accounting for effects of female size. Plotted for each of three successive matings is the relationship between male mass and the deviation from expected fecundity due to female size (i.e. the residuals following the regression of fecundity on female size). Results were similar for total egg production (see Table 5.2).

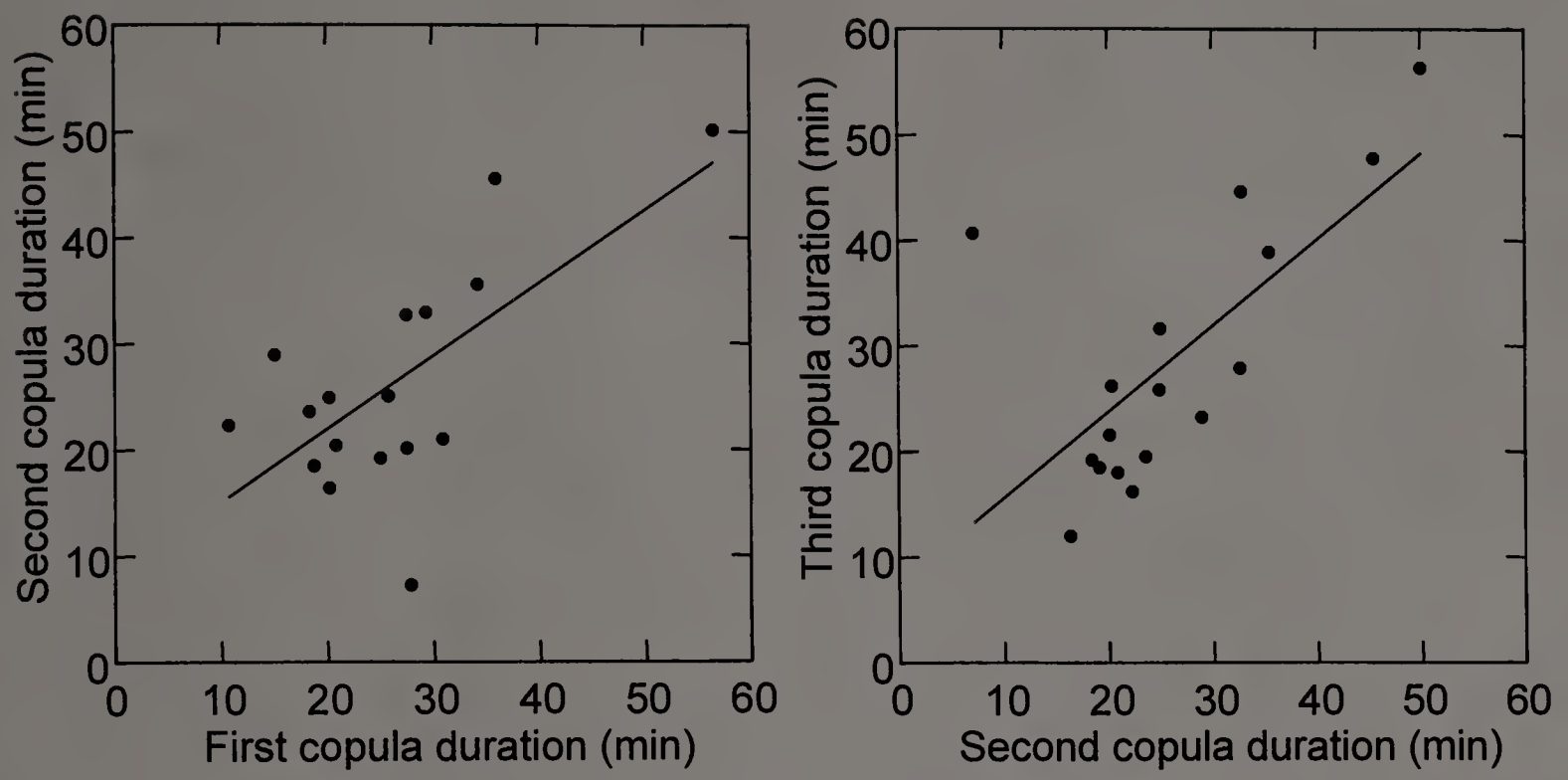


Figure 5.2. Relationship between successive copula durations.

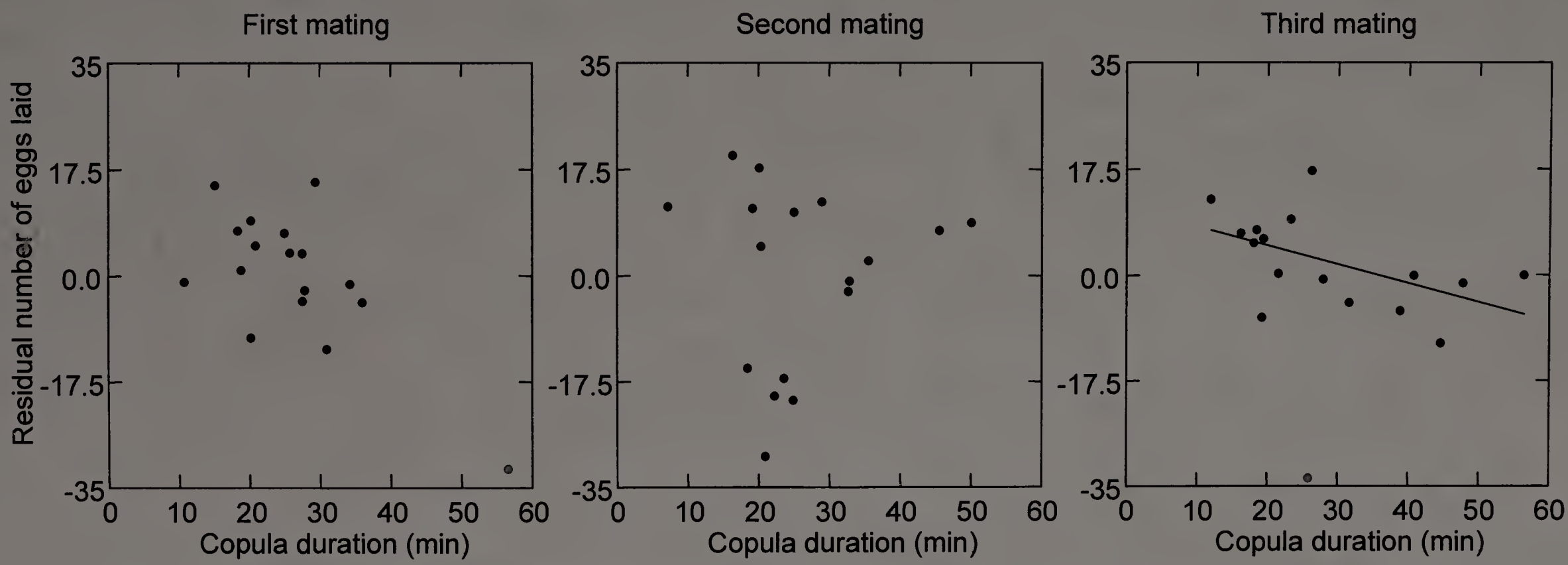


Figure 5.3. Relationship between copula duration and female reproductive output accounting for effects of female size. Plotted for each of three successive matings is the relationship between copula duration and the deviation from expected fecundity due to female size (i.e. the residuals following the regression of fecundity on female size). Results were similar for total egg production (see Table 5.4). The analyses for the first and third mating each exclude one outlier (gray point) in which the female laid zero or very few eggs.

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